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PRINCIPAL INVESTIGATOR: Andrew Berchuck, M.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, NC 27710

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14. ABSTRACT To better understanding the etiology of ovarian cancer, we have initiated a case-control study that considers genetic susceptibility, epidemiologic factors and somatic alterations. Subjects are interviewed in their homes and 1,100 cases and 1,000 controls have been accrued. Blood and cancer samples have been collected and molecular analyses of genetic polymorphisms have been performed. We have performed an Illumina array experiment with 1,536 haplotype tagging single nucleotide polymorphisms in about 150 candidate genes with an emphasis on DNA repair genes and found preliminary evidence of an association of this pathway with ovarian cancer risk. We also have played a leadership role in establishing an international consortium of groups to validate initial associations. A chemoprevention trial with levoneorel in chickens demonstrated a protective effect and we have shown that progestin mediated apoptosis in the ovarian epithelium is mediated by transforming growth factor-beta. In vitro data has suggested that vitamin D analogues may also represent appealing chemopreventives. A chemoprevention trial in chickens that incorporates both progestins and vitamin D analogues has been performed. These studies have the potential to increase our ability to identify high-risk women and to lead to the development of chemoprevention strategies that might decrease mortality from this disease.					
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Introduction

Ovarian cancer is the fourth leading cause of cancer deaths among women in the United States. There are three potential approaches to decreasing ovarian cancer mortality: screening and early detection, more effective treatment and prevention. All of these avenues should be explored, but we believe that prevention represents the most feasible approach. The rationale for prevention is derived from epidemiologic studies that have examined the relationship between reproductive history, hormone use and ovarian cancer. It has been convincingly demonstrated that reproductive events which reduce lifetime ovulatory cycles are protective. Although most women are unaware of this protective effect, those who use oral contraceptive pills for more than 5 years or have 3 children decrease their risk of ovarian cancer by greater than 50%. The biological mechanisms that underlie the association between ovulation and ovarian cancer are poorly understood, however.

Our multidisciplinary ovarian cancer research group has been actively involved in studies that seek to elucidate the etiology of ovarian cancer and to translate this knowledge into effective preventive strategies. Joint consideration of genetic susceptibility, reproductive/hormonal and other exposures, acquired alterations in oncogenes and tumor suppressor genes and protective mechanisms such as apoptosis is required to accomplish this goal. We have initiated a molecular epidemiologic study of ovarian cancer in North Carolina that focuses on the identification of genetic polymorphisms that affect susceptibility to ovarian cancer. Over 2,200 subjects have been accrued thus far in this case-control study. We have examined several polymorphisms and found that a polymorphism in the promoter of the progesterone receptor is associated with a decreased risk of endometrioid and clear cell ovarian cancers. We forged a collaboration relationship with Dr Georgia Chenevix-Trench in Australia, who also conducting a DOD funded case-control study of ovarian cancer. This collaboration was vitally important in allowing us to confirm these positive results prior to publication. This successful paradigm subsequently led to the establishment of an international ovarian cancer association consortium that includes 14 case-control studies. Dr. Berchuck serves as head of the steering committee of the consortium. The investigators have met every six months for the past two years and have collaborated on several validation studies of polymorphisms, including those in cell cycle genes and two papers have been accepted for publication. In addition, we will pool polymorphism data to increase statistical power to examine relationships with less common histologic types (eg. borderline and non-serous) and gene-gene and gene-environment interactions. Finally, the consortium is now collaborating on whole genome association studies that involve an initial analysis of 550,000 tagging snps. This initial phase will be followed by two stages in which the most promising candidate polymorphisms undergo validation.

We also are actively involved in development of chemopreventive strategies. We have performed a study in primates that suggests that the oral contraceptive has a potent apoptotic effect on the ovarian epithelium, mediated by the progestin component. In addition, in subsequent studies performed *in vitro*, we have induced apoptosis in epithelial cells treated with the progestin levonorgestrel. Progestin mediated apoptotic effects may be a major mechanism underlying the protection against ovarian cancer afforded by OCP use. This forms the basis for an investigation of the progestin class of drugs as chemopreventive agents for epithelial ovarian cancer. Initial studies to test the progestin levonorgestrel in an avian model of ovarian cancer have been undertaken and demonstrated a striking protective effect. In the present study, we are exploring the potential use of vitamin D compounds to enhance the apoptotic effect of progestins on the ovarian epithelium and to enhance the protection against ovarian cancer in the avian model. In addition, we are exploring the molecular pathways (most notably the TGF-beta pathway) that mediate progestin/vitamin D induced apoptosis in the ovarian epithelium. Finally, in an "idea project" we are exploring new pharmacologic approaches to targeting the progesterone receptor for ovarian chemoprevention.

Over the past eight years with support from the DOD Ovarian Cancer Research Program we have made considerable progress. This report focuses on the most recent progress in the past 12 months.

Body

Epidemiology and Tissue Core and Project 1: Genetic susceptibility to ovarian cancer

With the support of the Department of Defense Ovarian Cancer Research Program in 1998 we initiated a molecular epidemiologic study of ovarian cancer to work towards the goal of a better understanding of the etiology of ovarian cancer. Drs. Andrew Berchuck (Gynecologic Oncologist) and Joellen Schildkraut (Epidemiologist) are working together to lead this study. Our initial plan was to accrue frozen tumor tissue and blood from 500 epithelial ovarian cancer cases treated at Duke University, the University of North Carolina at Chapel Hill and East Carolina University. In addition, 500 age and race-matched control subjects were to be accrued and both cases and controls were to be interviewed by telephone regarding known risk factors for ovarian cancer. After funding to support this project was received from the Department of Defense in 1998 with Dr Berchuck as PI, additional funding was received to support this project in the form of an RO1 grant from the NCI with Dr Schildkraut as PI. The additional funding has allowed us to increase the scope of the study such that nurse interviewers are visiting the homes of all the cases and controls to administer the study questionnaire. Research subjects are now accrued from hospitals in a 48 county region of central and eastern North Carolina using a rapid case ascertainment mechanism established through the state tumor registry. Prior to initiating the study, we had to go through the process of IRB approval in each of the various hospitals involved. The second DOD Ovarian Cancer Program Project which began in 2002 provided funding to increase our accrual to 820 ovarian cancer cases and an equal number of controls. We have exceeded this accrual and over 1,100 women with ovarian cancer and 1,000 age and race-matched controls have been entered in the study and interviewed. The investigators have project meetings every month with all the research staff to review progress and address ongoing issues. We continue to obtain blood specimens from over 99% of our study subjects. All clinical, epidemiologic and molecular data are stored as they are obtained in a computerized database. Paraffin blocks of tumor tissue are also obtained and these tissues are being used to assess alterations in cancer causing genes such as p53, cyclin E and HER-2/*neu*. We are continuing to test the hypothesis proposed in the first DOD program project grant that alterations in specific genes may represent molecular signatures that characterize distinct molecular epidemiological pathways of causation of ovarian cancer.

During the study interview a thorough history of the menstrual cycle and reproductive experiences of the study participants is obtained from each subject assisted by the use a life-time calendar method. In addition, information on oral contraceptives and hormone replacement therapy is obtained. Data on the family history of cancer, other risk factors, and potential confounders is also collected. The interview takes 60-90 minutes to complete. The interactions between the nurses and subjects has been uniformly positive. The women with ovarian cancer are highly motivated to talk about their history and have a high level of interest in supporting a study aimed at increasing our understanding of the causes of ovarian cancer. They greatly appreciate the opportunity to talk with a nurse who is truly interested in hearing all the details of their life experience.

Although most of the genes responsible for dominant hereditary ovarian cancer syndromes (BRCA1/2, MSH2/MLH1) likely have been discovered, there is evidence to suggest that polymorphisms in other genes may also affect cancer susceptibility in a more weakly penetrant fashion. In project 1, we are examining the role of genetic susceptibility in the development of ovarian cancer. These studies focus on

genes involved in pathways implicated in the development of ovarian cancer. Since the effect of cancer susceptibility genes may be modified by other genes and exposures, he also will determine whether gene-gene and gene-environment interactions affect ovarian cancer susceptibility.

Presently, ovarian cancer risk stratification is not used to guide clinical surveillance or interventions in the vast majority of women, other than in rare individuals with BRCA1/2 or HNPCC mutations. This must change in the future if we are to decrease ovarian cancer incidence and mortality. The long term goal of our work is to identify a panel of ovarian cancer susceptibility polymorphisms that can be used in combination with known epidemiological risk factors such as parity and OC use to better stratify ovarian cancer risk. This would greatly facilitate implementation of screening and prevention strategies by allowing these to be focused on higher-risk populations.

Demographic and clinical features of ovarian cancer cases and controls in the North Carolina Ovarian Cancer Study

	Cases (N=1107)		Controls (N=998)		p-value
Age in years					
Mean (s.d)	55.3	(11.8)	54.9	(11.8)	0.440
median (range)	56	(19-83)	56	(20-75)	
	n	(%)	n	(%)	
Race					
Caucasian	943	(85)	802	(80)	0.007
African-American	135	(12)	170	(17)	
Other	29	(3)	26	(3)	
Tubal ligation					
No	823	(74)	637	(64)	<0.001
Yes	284	(26)	361	(36)	
Oral contraceptive use					
No	387	(35)	287	(29)	<0.001
≤ 12 months	201	(18)	169	(17)	
> 12 months	484	(44)	518	(52)	
User of unknown duration	34	(3)	23	(2)	
Livebirths					
0	234	(21)	132	(13)	<0.001
1	196	(18)	172	(17)	
2	348	(31)	366	(37)	
3	187	(17)	198	(20)	
>3	142	(13)	130	(13)	
Family History of Ovarian Cancer (1st degree)					
No	1051	(95)	967	(97)	0.011
Yes	55	(5)	28	(3)	

Family History of Ovarian
Cancer (2nd degree)

No	1058 (96)	960 (97)	0.278
Yes	48 (4)	34 (3)	

Tumor Behavior

Borderline	227 (21)
Invasive	872 (79)
Don't know	8

About 62% of cancers are serous and 60% are stage III/IV.

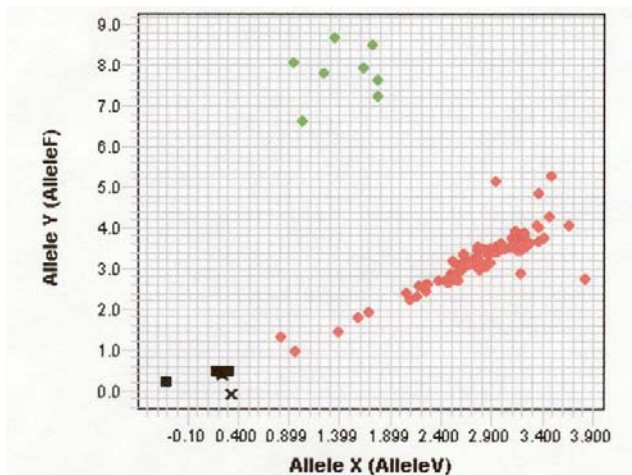
BRCA1/2: Since inherited BRCA1 or BRCA2 mutations strikingly increase ovarian cancer risk, polymorphisms in these genes could represent low penetrance susceptibility alleles. Prior studies of the BRCA2 N372H polymorphism suggested that HH homozygotes have a modestly increased risk of both breast and ovarian cancer. We have examined whether BRCA2 N372H or common amino acid-changing polymorphisms in BRCA1 predispose to ovarian cancer in the North Carolina ovarian cancer study. Cases included 312 women with ovarian cancer (76% invasive, 24% borderline) and 401 age- and race-matched controls. Blood DNA from subjects was genotyped for BRCA2 N372H and BRCA1 Q356R and P871L. There was no association between BRCA2 N372H and risk of borderline or invasive epithelial ovarian cancer. The overall odds ratio for HH homozygotes was 0.8 (95% CI = 0.4-1.5) and was similar in all subsets including invasive serous cases. In addition, neither the BRCA1 Q356R (OR = 0.9, 95% CI 0.5-1.4) nor P871L (OR = 0.9, 95% CI 0.6-1.9) polymorphisms were associated with ovarian cancer risk. There was a significant racial difference in allele frequencies of the P871L polymorphism ($P = 0.64$ in Caucasians, $L = 0.76$ in African Americans, $p < 0.0001$). In this population-based, case-control study, common amino acid changing BRCA1 and 2 polymorphisms were not found to affect the risk of developing ovarian cancer. These results were published in Clinical Cancer Research in 2003.

Progesterone receptor: In view of the protective effect of a progestin dominant hormonal milieu (OC use, pregnancy), progesterone receptor variants with altered biological activity might affect ovarian cancer susceptibility. A German group reported that an intronic insertion polymorphism in the progesterone receptor was associated with a 2.1-fold increased ovarian cancer risk. It subsequently was shown that this *Alu* insertion is in linkage disequilibrium with SNPs in exons 4 and 5. However, several subsequent studies by our group and others failed to confirm an association between these polymorphisms and ovarian cancer. In addition, there is little evidence that this complex of polymorphisms, termed PROGINS, alters progesterone receptor function.

More recently, sequencing of the progesterone receptor gene has revealed several additional polymorphisms, including one in the promoter region (+331G/A). The +331A allele creates a unique transcriptional start site that favors production of the progesterone receptor B (PR-B) isoform over progesterone receptor A (PR-A). The PR-A and PR-B isoforms are ligand-dependent members of the nuclear receptor family that are structurally identical except for an additional 164 amino acids at the N-terminus of PR-B, but their actions are distinct. The full length PR-B functions as a transcriptional activator and in the tissues where it is expressed it is a mediator of various responses, including the proliferative response to estrogen or the combination of estrogen and progesterone. PR-A is a transcriptionally inactive dominant-negative repressor of steroid hormone transcription activity that is thought to oppose estrogen-induced proliferation. An association has been reported between the +331A allele of the progesterone receptor promoter polymorphism and increased susceptibility to endometrial

and breast cancers. It was postulated that upregulation of PR-B in carriers of the +331A allele might enhance formation of these cancers due to an increased proliferative response.

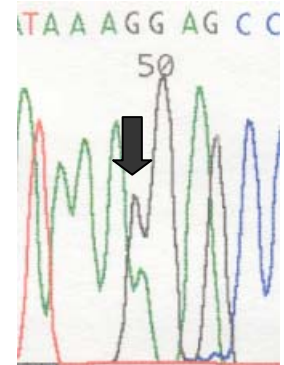
The +331G/A polymorphism in the progesterone receptor promoter was examined in cases and controls from the North Carolina Ovarian Cancer Study. A second, independent, case-control study from Australia (Dr. Chenevix-Trench) that is also funded by the DOD was examined to confirm associations seen in the North Carolina study. Data from the two studies was then pooled to increase statistical power. The +331G/A single nucleotide polymorphism in the promoter of the progesterone receptor was genotyped using a TaqMan assay. Allelic discrimination was performed using the MGB primer/probe TaqMan assay on the ABI Prism 7700 system. Some samples were sequenced using the ABI 3100 system to confirm the accuracy of the Taqman assay. The +331A allele was found in 59/504 (11.7%) Caucasian controls and the distribution of genotypes was in Hardy-Weinberg Equilibrium ($\chi^2 = 0.391$, $p = 0.53$). Only 1/81 (1.2%) African American controls and none of 67 African American women with ovarian cancer carried the +331A allele. In view of the rarity of the +331A allele in African Americans, these subjects were excluded from further analyses. The +331AA homozygotes were combined with heterozygotes in calculating odds ratios. The +331A allele was associated with a modest reduction in risk of ovarian cancer. Analysis by histologic type revealed that there was a slight trend towards protection against the common serous histologic type (OR = 0.80, 95% CI 0.49–1.29) but there was a more striking protection against endometrioid and clear cell cancers (OR = 0.30, 95% CI 0.09–0.97).



PR promoter polymorphism

(left) TaqMan assay (green = GA heterozygotes, red = GG homozygotes)

(right) GA heterozygote



Relationship between PR promoter polymorphism and ovarian cancer risk in histologic types of ovarian cancer

	PR +331 G/A Genotype					OR	(95% CI)	
	GG	AG	AA	AG/AA	(11.7%)			
Controls	445	58	1	59	(11.7%)	1.00	Reference	
Serous	244	26	0	26	(9.6%)	0.81	(0.50 -	1.32)
Mucinous	44	5	0	5	(10.2%)	0.80	(0.30 -	2.14)
Endometrioid	53	3	0	3	(5.4%)	0.43	(0.13 -	1.40)
Clear cell	23	0	0	0	(0.0%)	**		
Endometrioid/ clear cell	76	3	0	3	(3.8%)	0.30	(0.09 -	0.97)

In view of the potential for false-positive results in genetic association studies, confirmation was sought using an independent study population from Australia. The frequency of the +331A allele among Caucasian controls varied by less than 1% between the Australian and North Carolina studies. The Australian study was not a population-based case-control study and fewer data were available regarding risk factors. Nevertheless, the results of the Australian study were similar to those of the North Carolina study, with a modest overall protective effect that was most pronounced for endometrioid cancers (OR = 0.51, 95% CI = 0.17–1.53). The Breslow-Day chi-square test was used to assess homogeneity of the results from the two study populations. Analyses involving the combined data set showed a significant association between the +331A allele and decreased risk of endometrioid/clear cell cases. In combining the two studies there was a significant risk reduction (OR = 0.46, 95% CI = 0.23–0.92) ($P = 0.027$). These types represent 21% of invasive ovarian cancer cases. Endometriosis is known to increase risk of endometrioid and clear cell ovarian cancers, many of which may arise in ovarian deposits of endometriosis. In this study, endometriosis was associated with an increased risk of endometrioid/clear cell cancers (OR = 3.87, 95% CI = 2.09–7.17). The +331A allele appeared to be strongly protective against endometriosis (OR = 0.19, 95% CI 0.03 – 1.38), but this study was under powered to prove this conclusively.

The literature is fraught with false-positive association studies of genetic susceptibility polymorphisms, but several features mitigate the likelihood of this in the present study. First, the known protective benefit of progestins against ovarian cancer provides a preexisting biologic plausibility for the observed association. In addition, the finding that the +331A allele is protective against both endometrioid/clear cell cancers and their precursor lesion (endometriosis) also is supportive. Confirmation of the positive association obtained in North Carolina study by the Australian study also represents an additional critical validation step. Finally, unlike many polymorphisms that lack known functional significance, the +331A allele increases transcription of PR-B *in vitro*. This study provides evidence for the existence of low penetrance ovarian cancer susceptibility polymorphisms. If multiple polymorphisms are identified that either increase or decrease the risk of various histologic types of ovarian cancer, this might be used in the future for risk stratification that would facilitate screening and prevention strategies. The paper describing the relationship between the progesterone receptor promoter polymorphism and ovarian cancer was published in the December 2004 issue of *Cancer, Epidemiology, Biomarkers and Prevention*.

Because of the potential for false-discovery in genetic association studies we have conducted a meta-analysis of several ongoing case-control studies to confirm this association. The +331G/A PR polymorphism was genotyped in blood DNA of 4,614 Caucasian subjects from population-based, case-control studies in the North Carolina Ovarian Cancer Study, Australia (Dr Trench), Massachusetts (Dr Daniel Cramer at Harvard) and Southern California (Dr. Leigh Pearce at USC). There were 2,269 subjects with invasive or borderline ovarian cancer (1,430 serous, 538 endometrioid/clear cell, 301 mucinous) and 2,345 controls. We conducted a meta-analysis using a fixed effects model to produce summary Mantel-Hanzel odds ratios (OR) for the four studies. The +331A allele (AA or GA) was present overall in 10.6% (151/1,430) of serous cases, 5.4% (34/538) of endometrioid/clear cell cases, 10.3% (31/301) of mucinous cases and 10.7% (251/2,345) of controls. The distribution of alleles in the controls conformed to Hardy-Weinberg equilibrium. There was no relationship between the +331A allele and serous or mucinous ovarian cancers in any of the individual studies or in the meta-analysis (serous OR = 0.98, 95% CI 0.79 - 1.22, mucinous OR = 0.91, 95% CI 0.59 - 1.38). In contrast, a protective effect against endometrioid/clear cell cancers was noted in each study (North Carolina OR = 0.45, Australia OR = 0.66, Massachusetts OR = 0.69 and Southern California OR = 0.30) and in the meta-analysis of all four studies (OR = 0.56, 95% CI 0.39 - 0.82) ($p < 0.003$). These findings provide further evidence that the A allele of the +331G/A PR promoter polymorphism is carried by about 11% of Caucasians and is protective against endometrioid and clear cell ovarian cancers.

In the past year, an international ovarian cancer association consortium has been established to validate initial positive findings from individual studies. The first gene to be examined was the progesterone receptor. In this study, three PR single nucleotide polymorphisms (SNPs), for which previous data have suggested they affect ovarian cancer risk, were examined. These were PR promoter +331 C/T (rs10895068), PROGINS (rs1042838), and a 3' variant (rs608995). A total of 4,788 ovarian cancer cases and 7,614 controls from 12 case-control studies were included in this analysis. Unconditional logistic regression was used to model the association between each SNP and ovarian cancer risk and 2-sided p-values are reported. Overall, risk of ovarian cancer was not associated with any of the three variants studied. However, in histopathological sub-type analyses, we found a statistically significant association between risk of endometrioid ovarian cancer and the PROGINS allele (n=651, OR=1.17, 95% CI=1.01-1.36, p=0.036). We also observed evidence of an association between risk of endometrioid ovarian cancer and the +331C/T variant (n=725 cases; OR=0.80, 95% CI 0.62-1.04, p=0.100). These data suggest that while these three variants in the PGR are not associated with ovarian cancer overall, the PROGINS and the +331C/T variant may play a modest role in risk of endometrioid ovarian cancer. This data is in press in the British Journal of Cancer.

TGF- β receptor 1: Progestin induced apoptosis in the ovarian epithelium may be mediated by the TGF- β pathway, and this pathway is the target for chemopreventive efforts in Project 2. In project 1, we are investigating the possibility that TGF- β receptors are appealing candidate ovarian cancer susceptibility genes. A polymorphism in the TGF- β I receptor has been described that involves deletion of 3 alanines from a 9 alanine tract (T β R1(6A)). IT has been suggested that the 6A allele might predispose to the development of ovarian cancer and other cancer types. In addition, there is some evidence that the T β R1(6A) variant may be functionally significant and may confer an impaired ability to mediate TGF- β anti-proliferative effects.

In view of the evidence that the TGF β R1 polyalanine polymorphism may affect ovarian cancer risk, this polymorphism was genotyped in 588 ovarian cancer cases and 614 controls from the North Carolina study (see tables below). Significant racial differences in the frequency of the 6A allele were observed between Caucasian (10.7%) and African American (2.4%) controls (p<0.001). One or two copies of the 6A allele of the TGF β R1 polyalanine polymorphism were carried by 18% of all controls and 19% of cases, and there was no association with ovarian cancer risk (OR = 1.07, 95% CI 0.80 – 1.44). The odds ratio for 6A homozygotes was 1.81 (95% CI 0.65 – 5.06), but these comprised only 0.98% of controls and 1.70% of cases. The 6A allele of the TGF β R1 polyalanine polymorphism does not appear to increase ovarian cancer risk. Larger studies are needed to exclude the possibility that the small fraction of individuals who are 6A homozygotes have an increased risk of ovarian or other cancers. Polymorphisms in other members of the TGF- β family of ligands, receptors and downstream effectors also are appealing candidates. This data was communicated as an oral presentation at the 2004 meeting of the International Gynecologic Cancer Society in Scotland and was published in the journal Gynecologic Oncology in 2005 (see appendix).

Vitamin D Receptor pathway: High circulating levels of vitamin D may protect against ovarian cancer, since mortality rates are higher in northern latitudes where there is less sunlight. The most biologically active form of vitamin D, 1,25 (OH) $_2$ D $_3$, is produced in the skin through sunlight exposure and vitamin D exhibits significant antineoplastic properties. Several factors, both dietary and genetic regulate the production of 1,25 (OH) $_2$ D $_3$ from its precursor. A recent study suggested that about 22% of the variation may be accounted for by a putative major gene effect. Highly polymorphic loci involved in the metabolism and function of vitamin D include the vitamin D binding protein and vitamin D receptor genes. It has been suggested that a polymorphism in the vitamin D receptor gene involving a shared

haplotype that includes a change in the 3' untranslated region that alters transcriptional activity may be associated with increased prostate cancer risk. This has not been a uniform finding in all studies, however.

Vitamin D receptor polymorphisms are being examined in the North Carolina Ovarian Cancer Study to test the hypothesis that vitamin D biosynthesis in the skin can protect susceptible individuals from developing ovarian cancer and that genetic variation in the vitamin D pathway may modify this protective effect. Seven haplotype tagging SNPs that include three functional variants have been genotyped and analyses are being performed to examine the relationship between genetic variation, sunlight exposure and ovarian cancer risk.

BRAF polymorphisms

Mutations in the BRAF gene, which is part of the RAS pathway, occur in some borderline serous ovarian tumors. In view of this, polymorphisms in the BRAF gene are appealing candidates that might affect susceptibility to borderline ovarian cancer. Dr Chenevix-Trench organized a multicenter collaborative study of BRAF polymorphisms with each center contributing their borderline cases and matched controls. These polymorphisms were not found to affect susceptibility to borderline serous tumors and this data was published in the journal *Gynecologic Oncology* in 2005.

Androgen receptor

Androgens may play a role in the development of some ovarian cancers. Two trinucleotide repeat polymorphisms have been described in exon 1 of the androgen receptor (*AR*) gene that may affect its function. A highly polymorphic CAG repeat encodes a polyglutamine tract with alleles that vary from 5 – 34 repeats. A less polymorphic GGC repeat encodes a polyglycine tract and allele lengths vary from 6 - 20 repeats. Previous studies of ovarian cancer and *AR* repeat polymorphisms have been inconsistent. We analyzed CAG and GGC repeat length polymorphisms in the *AR* gene using data from a population-based case-control study of ovarian cancer that included 594 cases and 681 controls (see submitted manuscript in appendix). Repeat lengths for each individual were determined by fluorescent DNA fragment analysis using ABI GeneScan software. Change point models were used to determine appropriate repeat length cut points by race (African American vs. Caucasian). No relationship was observed between CAG repeat length and ovarian cancer among Caucasians. Among African Americans, a short CAG allele < 16 repeats was associated with a > 2-fold increase in ovarian cancer risk (age-adjusted OR = 2.8; 95% CI = 1.4 -5.9). No relationship with GGC polymorphism was observed among either race. These results suggest that the short CAG alleles (< 16 repeats) in *AR* increase ovarian cancer risk in African Americans. The failure to observe this relationship in Caucasians may be due to the rarity of such short CAG alleles in this population or could reflect racial differences in disease etiology. This work was published in 2007 in the journal *Cancer Epidemiology Biomarkers and Prevention* (see appendix).

Illumina array

In the last few years since our grant was funded, high throughput techniques for SNP genotyping have been developed. We have performed an Illumina array experiment that allowed us to genotype 1,536 SNPs in candidate genes in all of the samples from the North Carolina Ovarian Cancer Study. This included haplotype tagging SNPs for about 150 genes as well as nonsynonymous SNPs that result in amino acid changes. This experiment focuses on DNA repair pathways (53 genes) as well as the inflammation and hormonal pathways. The advent of this high throughput technology has allowed us to generate vastly more genotype data than we have generated in all the past years combined.

We adopted a modeling strategy that allows us to simultaneously calculate a measure of pathway-wide association and to identify the most likely associated SNPs. This approach involves a Bayesian model search strategy that implements an evolutionary Monte Carlo algorithm for inferring the likely model(s)

describing the association between ovarian cancer incidence and genetic variability in the DNA repair pathway while conditioning on age and prior breast cancer history. We calculate permutation p-values to measure the global association between variation in DNA repair pathway genes and ovarian cancer and estimate posterior inclusion probabilities to judge the importance of individual SNPs. Our global analyses of the increasingly focused case groups suggest a significant association between variation in the DNA repair pathway and serous invasive ovarian cancer among non-Hispanic whites (p-value = 0.035). Among serous invasive cases, we identify variants in CHEK2, TP53, MSH3, LIG4, RAD52, XRCC5 and NBS1, among other genes, that have high posterior probabilities of association. While this analysis is suggestive of associations amongst the variants we interrogated and provides some guidance as to the candidates driving those associations, further study and evaluation will be required to pinpoint those associations. Currently, several of these are being evaluated by the OCAC and several more will be proposed for evaluation in the next wave.

Ovarian Cancer Association Consortium

Although case-control studies of some polymorphisms have reported positive associations, these generally have not been confirmed in subsequent studies. Groups from the US, UK and Australia met in at Cambridge University in April 2005 to review results of various ongoing ovarian cancer association studies. There was a consensus that many of the challenges inherent in this field can best be addressed by collaborative efforts. In view of this, the group elected to establish an ovarian cancer association consortium (OCAC). Dr. Berchuck successfully applied to the Ovarian Cancer Research Fund for a \$900,000 grant to fund the first three years of biannual meetings and other activities, and serves as the head of the steering committee. Dr Georgia Chenevix-Trench, who also is funded by the DOD Ovarian Cancer Research Program also is a member of the steering committee.

The aims of the consortium are an outgrowth of the North Carolina and Australian DOD funded studies and reflect the successful translation of the DOD funding into a continued and expanded effort. The second meeting of the ovarian cancer association consortium took place in Salt Lake City in October 2005 in concert with the American Society of Human Genetics annual meeting. All groups conducting ovarian cancer case-control studies of genetic polymorphisms were invited to join the consortium. Presently participants include, Duke, USC, Australia, Cambridge, London, Denmark, Poland/NCI, Harvard, Yale, Pittsburgh, Hawaii, Stanford, Mayo and Moffitt. In 2007 meetings were held in London in April and in October in Los Angeles. The aims of the consortium are listed below.

Aim #1 - To develop an ovarian cancer association consortium (OCAC) that is dedicated to working together to identify and validate common low penetrance ovarian cancer susceptibility polymorphisms. The OCAC will meet each fall in concert with the American Society of Human Genetics meeting, and an annual spring meeting will be hosted by an OCAC member institution. This will provide the opportunity for face-to-face interactions that are critically important in sustaining the momentum of the OCAC.

Aim #2 – To perform a comprehensive review of the existing ovarian cancer susceptibility polymorphism literature. This effort will produce a review article and will serve as a marker of the state of the field as the OCAC begins its work.

Aim #3 – To determine whether polymorphisms in the progesterone receptor affect ovarian cancer risk. Polymorphisms in the progesterone receptor (PR) gene have been the most frequently examined. Several studies have suggested that polymorphisms in this gene affect risk, but not all studies have not confirmed these findings. The OCAC members will genotype PR polymorphisms in several thousand cases and controls and the data will be analyzed centrally to resolve the issue of whether PR variants

affect ovarian cancer risk. (This work has been accomplished and was presented by Dr Berchuck as an oral presentation at the 2006 meeting of the International Gynecologic Cancer Society in Los Angeles.

Aim #4 – To examine associations between other promising candidate genetic variants and risk of ovarian cancer. In keeping with the goal of the OCAC to provide definitive evidence of genetic associations, the most promising candidate variants being studied by OCAC members will be genotyped in a collaborative manner as described above for the progesterone receptor.

The OCAC has most recently examined seven candidate single nucleotide polymorphisms (SNPs) for which there was prior evidence of association in single-institution studies of ovarian and/or breast cancer (AUKRA F31I, BRCA2 N372H, RB1 intron 17 rs2854344, CDKN2A rs2811712, SRD5A2 V89L, CASP8 D302H and TGFB1 L10P). These SNPs were genotyped in fourteen case-control studies of Caucasian subjects from the US, UK, Europe and Australia that included 4,624 invasive epithelial ovarian cancer cases and 8,113 controls. Genotyping was performed using a common quality-control 96 well plate of DNAs and screening for duplicate concordance, minimum call rates, and Hardy-Weinberg equilibrium. Data were analyzed using logistic regression to calculate age- and study-adjusted odds ratios (OR's) and 95% confidence intervals (CI's) assuming a variety of genetic models. A significant association was found for RB1 rs2854344 with a per-allele OR of 0.88 (95% CI 0.79-1.00; $p=0.041$) including all studies. An association was also observed for AUKRA F31I (per-allele OR 1.10, 95% CI 1.01-1.20, $p=0.027$), when one study was excluded which was causing significant heterogeneity. No association with ovarian cancer risk was observed with the other five SNPs (BRCA2 N372H, CDKN2A rs2811712, SRD5A2 V89L, CASP8 D302H, and TGFB1 L10P); given the large sample size, these null results are also informative. No differences in associations were observed by stage or histologic type. Through analysis of the world's largest collection of ovarian cancer cases and controls, the OCAC has replicated the association of two candidate SNPs with ovarian cancer risk. The SNP in RB1 is particularly interesting as it has been shown to exhibit a protective association in both breast and ovarian cancer. These data demonstrate the feasibility of the OCAC working together collaboratively to identify common polymorphisms that affect ovarian cancer susceptibility. The goal of the OCAC is to identify a panel of susceptibility polymorphisms that can be used to better stratify ovarian cancer risk. This would greatly facilitate screening and prevention strategies by allowing these to be focused on higher-risk populations. This work will be presented at the 2008 annual meeting of the Society of Gynecologic Oncologists in Tampa, Florida and has been submitted for publication.

Aim #5 – To assign groups to write additional grant proposals that focus either on specific molecular pathways using a comprehensive approach or methodological issues for association studies. The groups in the ovarian cancer association consortium are funded to study specific genes and/or gene pathways. This includes various steroid hormone, DNA repair and inflammation related pathways as well as others. The goal will be to assign groups to seek additional funding to study these pathways in the OCAC. In addition, the group will be uniquely positioned to study methodological issues related to genetic association studies and the statistical geneticists in the group will have the opportunity to apply for funding to use OCAC data for this purpose.

Aim #6 – To examine the interaction between major epidemiological risk factors and genetic polymorphisms. Because of the moderate size of most ovarian cancer association studies it has not been possible to perform analyses of gene-environment interactions. The OCAC will establish a common data sheet that includes basic information relating to major epidemiological risk factors. This will focus mainly on family history and reproductive risk factors. Central analyses will be performed to examine interactions between factors such as OC use, genetic polymorphisms and ovarian cancer risk.

Relationship between epidemiological risk factors and somatic alterations in ovarian cancer

One of the goals of the original DOD program project grant that was funded in 1997 was to examine whether somatic alterations in ovarian cancer define distinct disease subsets. We had previously found that overexpression of mutant TP53 was associated with high life-time ovulatory cycles in ovarian cancer. More recently, using the cases and controls in the North Carolina Ovarian Cancer Study, we have examined whether cyclin E overexpression defines an etiologically distinct subgroup of ovarian cancer. Overexpression of cyclin E is one of the most frequent molecular alterations described thus far in advanced ovarian cancers. We analyzed data from 413 invasive epithelial ovarian cancer cases, 123 tumors of low malignant potential and 629 controls. Cyclin E protein overexpression was assessed using immunohistochemistry. Case-control comparisons showed that months of pregnancy and oral contraceptive use were inversely associated with risk of ovarian cancers that overexpress cyclin E but not with those that lacked expression. There was a dose response relationship between lifetime ovulatory cycles (LOCs) and ovarian cancer that overexpressed cyclin E (OR = 1.8, 95% CI 1.1 – 3.0 for moderately high LOCs (265 – 390 cycles) and OR = 2.7, 95% CI 1.6 – 4.5 for high LOCs (>390 cycles) as compared to low LOCs (<265 cycles)) but no relationship was seen with cancers that lacked overexpression. The most important components of the LOC variable contributing to the differences in the association with the cyclin E subgroups of ovarian cancer were months of OC use and months pregnant. This suggests that cyclin E overexpression is a molecular signature characteristic of ovarian cancer cases that arise via a causative pathway that involves ovulation-induced alterations.

Project 2: Chemoprevention of Ovarian Cancer

We have previously reported in prior updates a novel finding which has great potential for translating into a pharmacologic **chemopreventive** approach to ovarian cancer that has both enhanced efficacy and decreased toxicity. In ovarian cancer cell lines as well as immortalized cell cultures derived from the normal human ovarian epithelium, we demonstrated that the combination of a progestin and vitamin D act synergistically to inhibit cell viability by inducing apoptosis. We therefore hypothesized that progestins and vitamin D target the early steps of carcinogenesis in the ovarian epithelium by activating pathways leading to apoptosis, thereby decreasing dysplastic ovarian epithelial cells and resulting in effective cancer prevention. In addition, we hypothesized that the combination of two preventive agents such as progestin plus vitamin D will be a more potent ovarian cancer preventive than either agent used alone, making it possible to lessen the dose of each in order to achieve optimal chemoprevention, while minimizing side effects.

We performed studies aimed at elucidating the biologic mechanisms that underlie the synergistic effect of the vitamin D/progestin combination. These studies included examining the effects of the progestin/vitamin D combination on TGF-beta signaling events, apoptosis and the cell cycle as well as determining whether progestin might alter the actual pharmacology of vitamin D by inhibiting its degradation. In experiments performed *in vitro* in immortalized cells derived from normal human ovarian epithelium (HIO-118V) as well as in the ovarian cancer cell line OVCAR-3, we observed that progestin decreases production of TGF-beta-1, similar to what we have observed in primates *in vivo*. In addition, the addition of a second agent (the phytoestrogen genistein) further decreased production of TGF-beta-1, and, when combined with vitamin D and progestin, completely abrogated TGF-beta-1 production, concomitant with a dramatic increase in cell death. However, since the combination of vitamin D and progestin produced an effect on TGF-beta-1 which was intermediate between that of each agent administered individually, we concluded that the synergistic effects of the combination of progestin and vitamin D are unlikely to be related solely to effects secondary to TGF-beta-1, although TGF-beta signaling events may be involved.

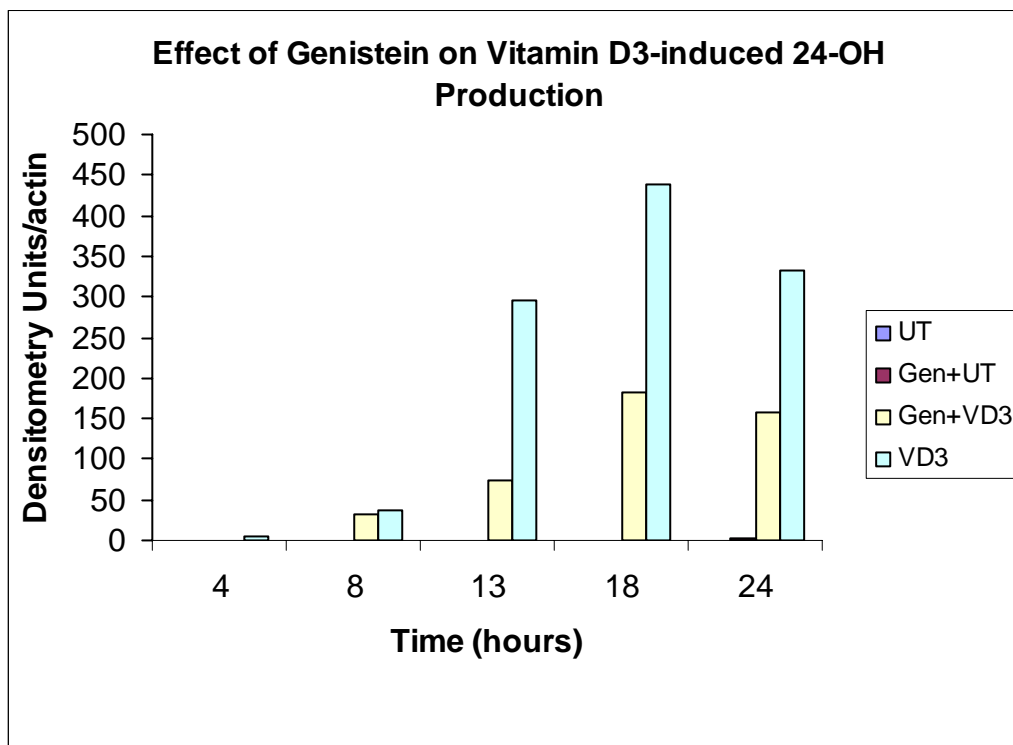
The vitamin D metabolizing enzyme 24 hydroxylase (24-OH, or CYP24) converts the active form of vitamin D (1,25 (OH)₂ D) to an inactive form via 24 hydroxylation. Of note, many cancer cells overexpress 24-OH, rendering them resistant to the effects of vitamin D. Moreover, 24-OH is normally induced in cells in response to vitamin D. This serves to inhibit unbridled vitamin D effects and to turn off vitamin D once it has achieved its biologic effect. Agents such as genistein and ketoconazole are known to cause degradation of 24-OH. We examined the effect of progestin on 24-OH in cells derived from the ovarian epithelium. We demonstrated that progestin causes degradation of 24-OH (please see induced double band for 24-OH in western blot shown in last year's report), an effect enhanced by the addition of genistein. This has not been previously shown, but may explain in part the synergy associated with the progestin-vitamin D combination. Namely, by inhibiting vitamin D's degradation via inhibition of 24-OH, the active form of vitamin D has a longer local biologic half life, and thus cellular effect.

Characterization of the Impact of Progesterone on Vitamin D 24-Hydroxylase on the Ovarian epithelium

As our data suggested a unique role of progesterone as a potentially negative regulator of 24-OH (CYP-24), we have continued to pursue these studies to focus on potential mechanisms. In addition, we have broadened the scope of studies to determine whether the novel observation that progesterone impacts CYP24 is an effect specific to the ovarian epithelium or whether this effect also occurs at other organ sites that express the progesterone receptor. Thus, we have widened the scope of experiments to include

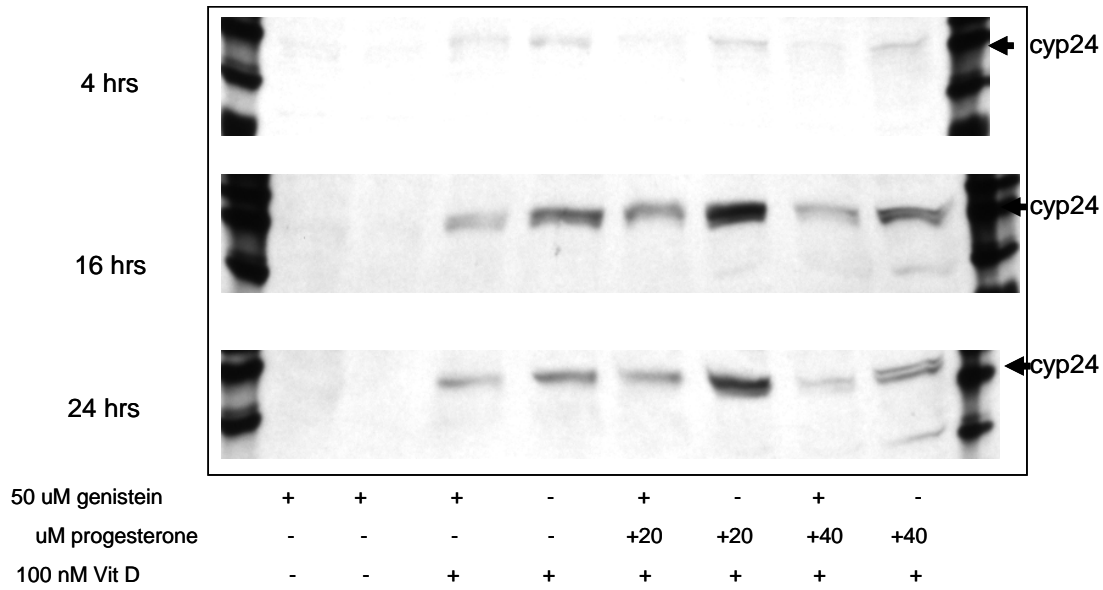
cancers of the endometrium and breast. Our strategy has included time-course assays, designed to identify optimal time points for maximum CYP24 production in response to vitamin D. In addition, we have performed MTS assays to examine the effects of progestin and vitamin D combinations. Expression of CYP24 has been examined primarily by western blot, with RT-PCR used to confirm our western blot findings. In addition, we have examined how preincubation with genistein prior to adding vitamin D would affect CYP24 production in ovarian and endometrial cancer cells treated with vitamin D alone or with progestin.

Our first series of experiments showed that CYP24 is upregulated in a time-dependent manner according to both western blot and RT-PCR. According to RT-PCR, CYP24 mRNA expression peaks at 4 hours; western blot indicates peak CYP24 production at 18 hours. In addition, we see that preincubating cells with 50 μ M genistein for 24 hours inhibits CYP24 production 18 and 24 hours after vitamin D stimulation.



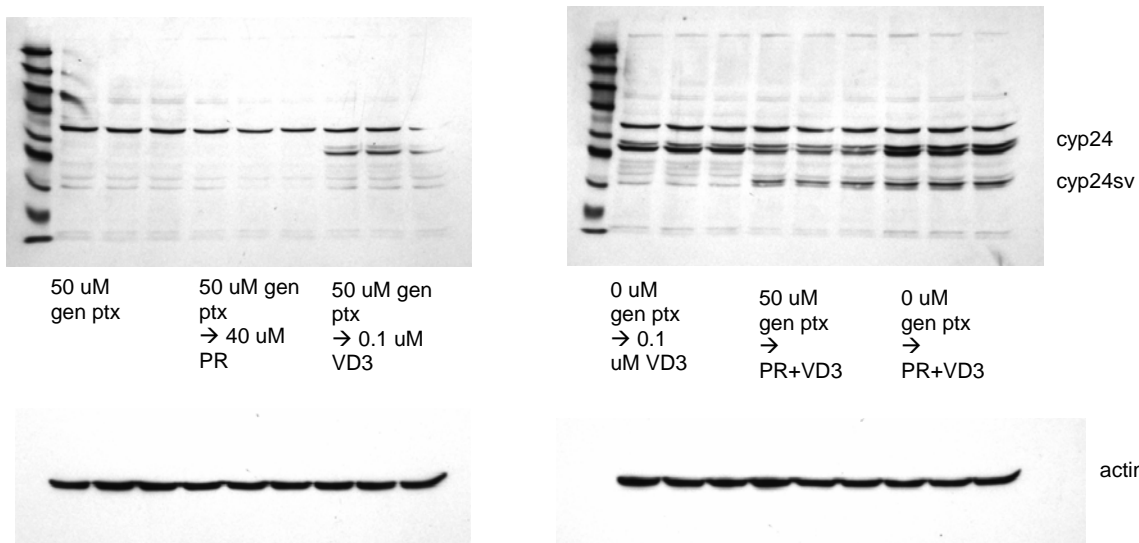
Of note, in some experiments we have observed a biphasic effect of progestin on vitamin D-induced CYP24. Shown below at 4, 16 and 24 hours we demonstrate that 20 μ M progesterone may increase CYP24 production; however, the resolution of this gel obscures the double band which may mean that it is, in fact, inactivating the protein. This is more clearly seen at 40 μ M. At all time points, genistein inhibits the production of CYP24.

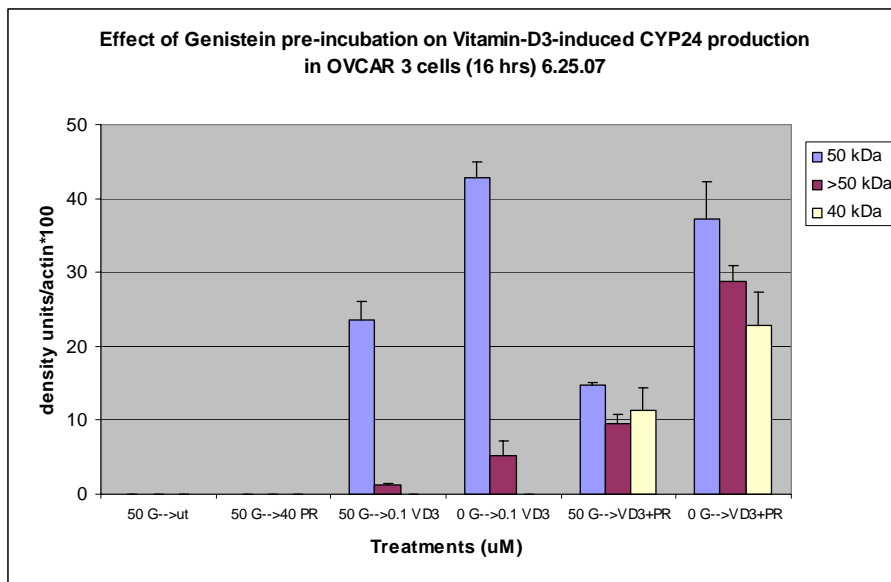
Genistein preincubation enhances progesterone inactivation of 24-OH



When this experiment was repeated at 16 hrs in triplicate, we see that genistein pretreatment enhances what appears to be degradation of CYP24. Seen more clearly is the possible splice variant at 40 kDa in the last three lanes. (see below)

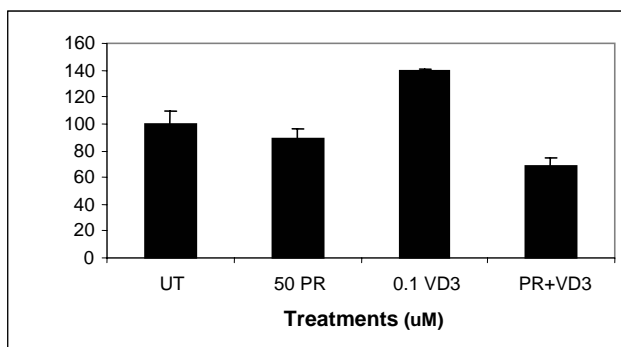
Effect of Genistein pre-incubation on Vitamin-D3-induced CYP24 production in OVCAR 3 cells (16 hrs)



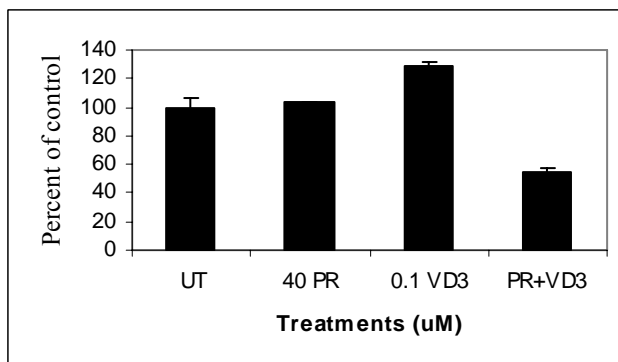


Does the Impact of Progesterone on Vitamin D 24-Hydroxylase on the Ovarian epithelium extend to other cell lines?

Cell viability via MTS assay was assessed in a number of human endometrial cancer cell lines by treating with progesterone, 1,25 (OH)₂ D₃, or the combination of progesterone and 1,25 (OH)₂ D₃. At doses of progesterone or Vitamin D that had modest or negligible effects on overall cell viability when either agent was administered alone, a marked and statistically significant decrease in cell viability was observed in the HEC1A and RL95-2 cell lines when the two agents were combined ($p < 0.01$). Analysis of the cell viability data using the CalcuSyn program (Biosoft) demonstrated that the combination of progestin and Vitamin D have synergistic effects on cell death, relative to the effect of either agent alone.



HEC1A



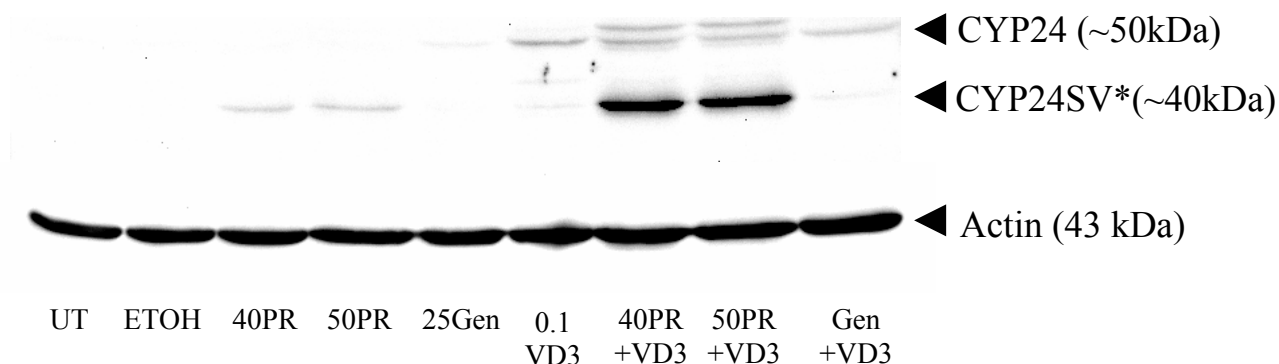
RL95-2

Effect of Progesterone (PR) and 1,25(OH)₂D₃ (VD3) on cell viability (MTS Assay). UT= untreated. Y- axis shows percent of untreated control.

MTS experiments were repeated in the breast cancer lines, MCF-7 and T47D which both express progesterone receptor (PR). At 30 uM progesterone+100 nM VD3, there was a modest (10%) decrease in cell proliferation in MCF-7 and T47D cells when compared with those cells which had been treated with progesterone alone (data not shown).

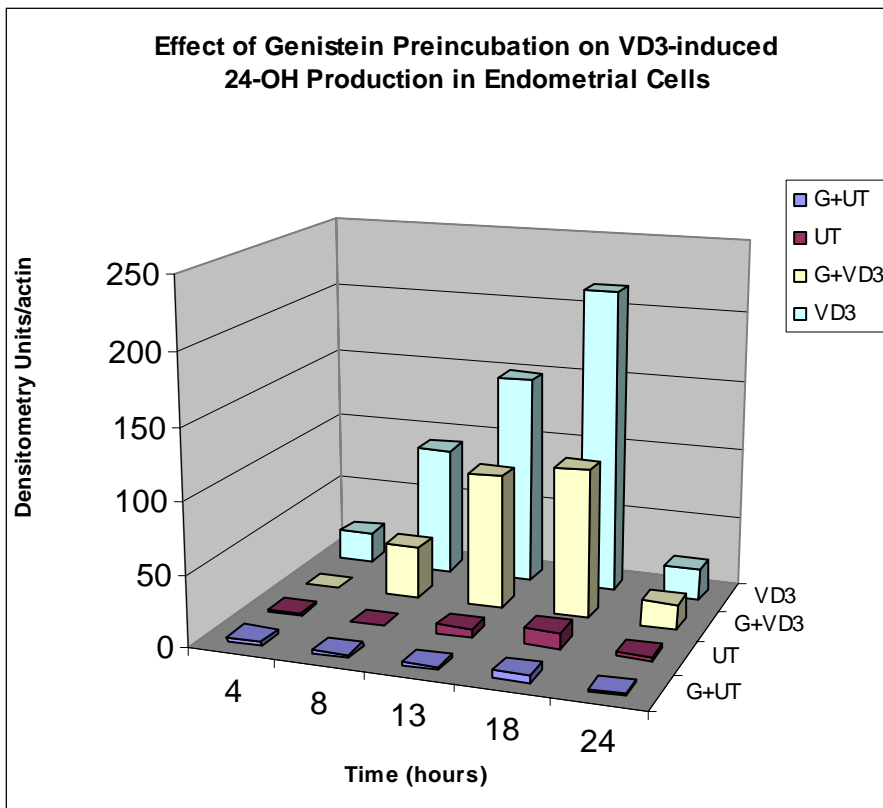
Progestin Inhibits Vitamin D 24 hydroxylase (CYP24) in endometrial cancer cell lines: To study potential mechanisms that may underlie the synergistic effect of progestins and vitamin D in the endometrium, we examined whether progestin modifies expression of CYP24 as we observed in the ovarian cancer cells. As is shown in the western blot below, CYP24 is induced by vitamin D, as expected. The amount of vitamin D-induced CYP24 protein is decreased in the presence of genistein (lane in far right). Progesterone causes both a decrease in the amount of CYP24 protein, as well as degradation of CYP24 protein as manifested by the appearance of a new band (second and third lanes from right, at MW 40kDa) which may be a splice variant of CYP24.

Western Blot for CYP24; RL95-2 Endometrial Cancer Cell



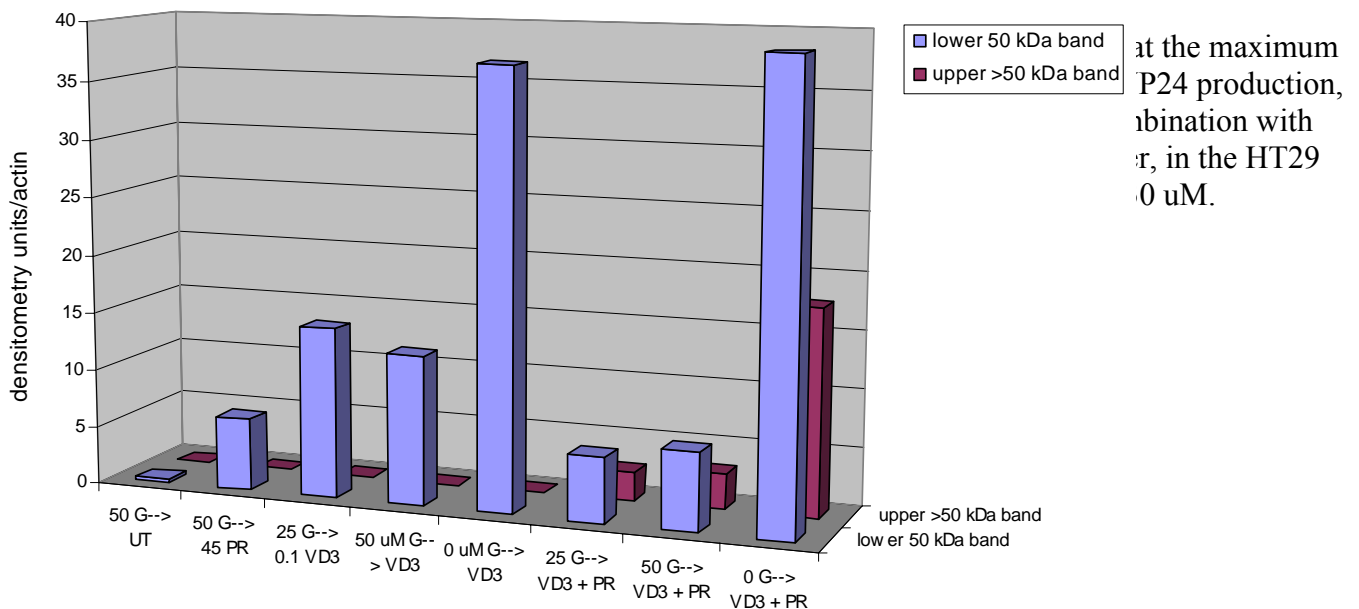
Treatments (uM); Gen = Genistein; PR = progesterone; VD3 = 1,25 (OH)₂ D₃;

With these results, we repeated similar time course experiments as in the ovarian lines, found that CYP24 was similarly upregulated in a time-dependent manner and also inhibited by genistein. We see below that vitamin D3 treatment induces a peak CYP24 response at 18 hours. Genistein most effectively blocks this response at 13 hours.



We next repeated pretreatment of genistein on progesterone and vitamin D3-treated cells at 8 hours and saw a similar enhancement of CYP24 inhibition when cells were treated with genistein. Again we see the cleavage of the 50 kDa band into two components with progesterone treatment.

**Effect of Genistein Preincubation on VD3 and PR-induced CYP24 production in treated RL95-2 cells
8 hr incubation**

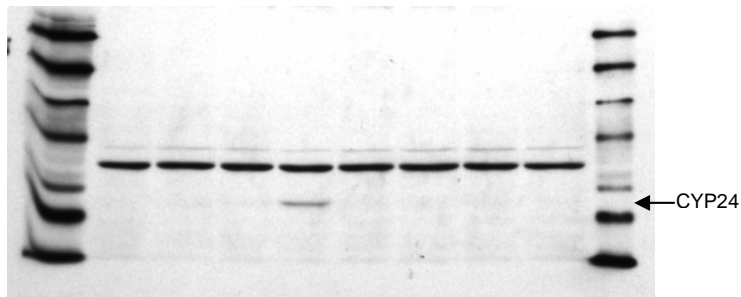


We performed similar experiments on the breast cancer lines, MCF-7 and T47D wild type (WT), finding that CYP24 was maximally induced at 16 hours in response to vitamin D. Our initial experiments indicated that progesterone induced a complete block of CYP24 production at 20 μ M, but inhibition as

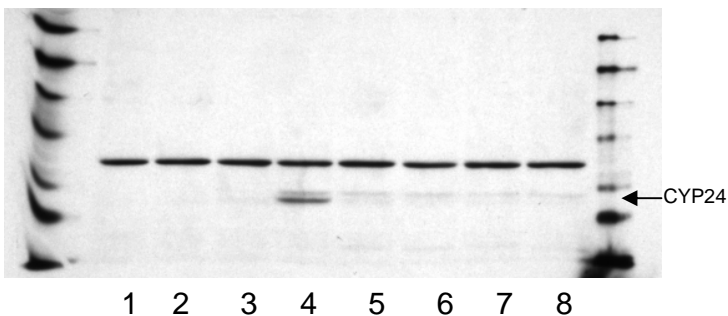
low as 1 μ M. We had previously observed that this dose, when combined with vitamin D, inhibited proliferation in MTS assays.

Effect of PR on Vitamin D3-induced 24OH Production (16 hr)

T47-D



MCF-7



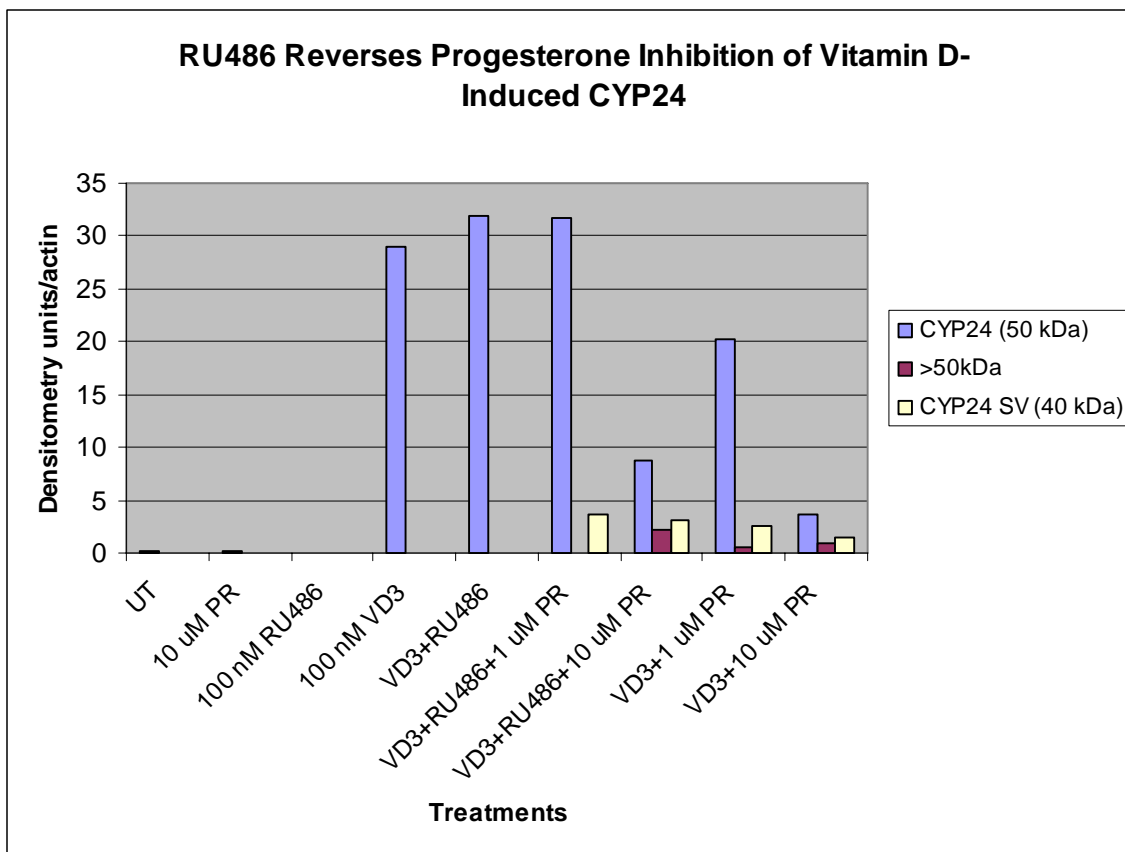
Legend

1. UT
2. 30 μ M PR
3. 10 nM VD3
4. 100 nM VD3
5. 20 PR + 10 VD3
6. 30 PR + 10 VD3
7. 20 PR + 100 VD3
8. 30 PR + 100 VD3

Are PR effects on 24-OH progesterone receptor-mediated?

We have begun to perform experiments using T47D cells that lack one or both progesterone receptor (PR) isoforms (T47D Y- PR negative; T47D YA – PR A isoform expression only; T47D YB - PR B isoform expression only) to see if we can determine whether the effect is mediated by PR, and if so, if one or both isoforms are required.

In addition, we are performing experiments using RU486 to block PR, and are planning experiments in conditions that include actinomycin D to determine whether some effects are nongenomic. Our preliminary data demonstrate that the PR antagonist RU486 abrogates the inhibitory effect of 1 μ M progesterone on vitamin D-induced CYP-24 by western blot (see below).



Summary of In Vitro evidence

We have previously shown that when cells derived from the ovarian surface epithelium are treated with a combination of progestin and vitamin D, it causes a synergistic decrease in cell proliferation which ultimately results in cell death. Perhaps most significant is our unique observation that progestin may cause the degradation of vitamin-D stimulated 24-OH, a candidate oncogene which metabolically inactivates the active form of vitamin D. Therefore, we have focused our efforts to understand the mechanism of how progestin may work to negatively affect upregulation of 24-OH. To accomplish this, we have broadened our scope to see whether different types of cancers respond similarly to ovarian cancers. We have discovered that in the ovarian, endometrial and breast cancer cell lines tested, there is a time-dependent vitamin D upregulation of CYP24. Progestin appears to cause cleavage of CYP24 at 50 kDa with varying amounts of what might be a splice variant at ~ 40 kDa. This effect is most pronounced in T47D WT which is progesterone receptor (PR)-rich. Genistein, a known inhibitor of CYP24 in prostate lines, augments progestin in reducing vitamin D-induced CYP24 production when ovarian cancer OVCAR3 cells and endometrial cancer RL95-2 cells are preincubated with genistein.

Looking forward, we will further study the mechanism of how progestin exerts its CYP24 inhibitory effects to determine whether this effect is PR-mediated and whether the pathway is genomic or nongenomic. In this way, we may provide further mechanistic evidence of how the combination of progestin and Vitamin D may act as a chemopreventive of ovarian cancer. Finally, we are processing tissues from a trial in chickens, designed to evaluate progestins and vitamin D as ovarian chemopreventives. In addition to tumor endpoints, we will plan to examine the impact of vitamin D, progestin, and the combination of expression of CYP24 in the normal chicken ovary and oviduct, as well as chicken ovarian and oviductal carcinomas.

Key research accomplishments

- 1) We have accrued over 2,100 subjects to a prospective, population-based, case-control study of ovarian cancer in North Carolina. Blood and tissue samples and epidemiologic data have been accrued as well. Analyses of genetic susceptibility polymorphisms and molecular epidemiologic signatures are ongoing.
- 2) An international ovarian cancer association consortium (OCAC) has been created to work towards an understanding of the role of genetic polymorphisms in ovarian cancer susceptibility. Dr Berchuck serves as head of the OCAC steering committee.
- 3) The +331G/A polymorphism in the progesterone receptor is protective against endometrioid/clear cell ovarian cancers and this has been confirmed by the international ovarian cancer association consortium.
- 4) Short alleles of the androgen receptor gene CAG repeat polymorphism increase risk of ovarian cancer in African American women in North Carolina.
- 5) A pathway analysis performed using the Illumina SNP array platform suggests an association between common genetic variations in DNA repair genes and ovarian cancer susceptibility.
- 6) We have shown that progestins markedly activate TGF- β signaling pathways in the ovarian epithelium in primates, and that these effects are highly associated with apoptosis. We are now performing studies *in vitro* designed to characterize the complex biologic effects of progestins and vitamin D analogues on apoptotic and TGF- β signaling pathways in ovarian epithelial cells. These findings will provide guidance in conducting a chemopreventive trial in chickens with these agents.
- 7) In view of *in vitro* evidence suggesting that there may be synergy with respect to ovarian cancer chemoprevention between progestins vitamin D analogues, and this concept has been tested in the context of a chemoprevention trial in chickens.
- 8) We have discovered that in the ovarian, endometrial and breast cancer cell lines tested, there is a time-dependent vitamin D upregulation of CYP24.

Reportable outcomes

- 1) The +331G/A polymorphism appears to be protective against endometrioid and clear cell ovarian cancers.
- 2) An international ovarian cancer association consortium has been formed that will work together to validate associations between genetic polymorphisms and risk of the disease.
- 3) Combinations of progestins and vitamin D may act in an additive fashion to decrease growth of ovarian cancer cells and is being studied in the context of a chemoprevention trial in chickens.

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Conclusions

The studies initiated by our program have the potential to enable us to define a moderate risk population based on epidemiologic and molecular genetic risk factors and to develop chemopreventive strategies designed to decrease ovarian cancer incidence and mortality.

With regard to ovarian cancer risk stratification, currently this is not used currently used clinically in the general population. This must change in the future if we are to decrease ovarian cancer incidence and mortality. The studies of genetic polymorphisms and molecular epidemiology initiated by our group are melding with those of other groups in the formation of an ovarian cancer association consortium. The long term goal is to identify a panel of ovarian cancer susceptibility polymorphisms that can be used in combination with known epidemiological risk factors to better stratify ovarian cancer risk. This would

greatly facilitate implementation of prevention strategies by allowing these to be focused on higher-risk populations.

There is reason to believe that chemoprevention of ovarian cancer can contribute to a decline in mortality. The investigations ongoing in our program that include both *in vitro* experiments and chemoprevention trials in chickens are paving the way towards implementation of progestins and vitamin D analogues in this context. This may represent the best approach to decreasing ovarian cancer deaths in the 21st century.

Appendices

Trinucleotide Repeat Polymorphisms in the Androgen Receptor Gene and Risk of Ovarian Cancer

Joellen M. Schildkraut,¹ Susan K. Murphy,² Rachel T. Palmieri,⁷ Edwin Iversen,³ Patricia G. Moorman,¹ Zhiqing Huang,² Susan Halabi,⁴ Brian Calingaert,⁶ Alison Gusberg,² Jeffrey R. Marks,⁵ and Andrew Berchuck²

¹Department of Community and Family Medicine; ²Division of Gynecologic Oncology, Department of Obstetrics and Gynecology;

³Institute of Statistics and Decision Sciences; Departments of ⁴Biostatistics and Bioinformatics and ⁵Surgery;

and ⁶Comprehensive Cancer Center, Duke University Medical Center, Durham, North Carolina; and

⁷Department of Epidemiology, The University of North Carolina, Chapel Hill, North Carolina

Abstract

Introduction: Androgens may play a role in the development of ovarian cancers. Two trinucleotide repeat polymorphisms have been described in exon 1 of the androgen receptor (AR) gene that may affect its function. Previous studies of ovarian cancer and AR repeat polymorphisms have been inconsistent. **Methods:** We analyzed CAG and GGC repeat length polymorphisms in the AR gene using data from a population-based case-control study of ovarian cancer that included 594 cases and 681 controls. Repeat lengths were determined by fluorescent DNA fragment analysis using ABI GeneScan software. Change point models were used to determine appropriate repeat length cutoff points by race (African American versus Caucasian) for both the shorter and longer CAG and GGC repeats.

Results: No relationship was observed between CAG repeat length and ovarian cancer among Caucasians.

Among African Americans, having a short repeat length on either allele was associated with a 2-fold increase in ovarian cancer risk (age-adjusted odds ratio, 2.2; 95% confidence interval, 1.1-4.1). Having short CAG repeat lengths for both alleles was associated with a 5-fold increased risk for developing ovarian cancer (age-adjusted odds ratio, 5.4; 95% confidence interval, 1.4-1.7). No relationship with the GGC repeat length polymorphisms was observed.

Conclusion: These results suggest that having a short CAG repeat length in AR increases ovarian cancer risk in African Americans. The failure to observe this relationship in Caucasians may be due to the rarity of such short CAG alleles in this population or could reflect racial differences in disease etiology. (Cancer Epidemiol Biomarkers Prev 2007;16(3):473-80)

Introduction

It has been suggested that androgens may play a role in the development of ovarian cancer (1, 2). Androgen is produced by ovarian theca lutein cells and androgen receptors (AR) are found in the normal surface epithelium of the ovaries. Most ovarian cancers express AR and antiandrogens inhibit ovarian cancer growth (3-7). Epidemiologic studies also support a role of androgen in ovarian cancer in which increasing waist-to-hip ratio (8, 9) and polycystic ovarian syndrome (9, 10), which may be correlated with elevated androgen levels in women, have been associated with increased risk of ovarian cancer. In one study, higher levels of serum androstenedione were reported among women diagnosed with ovarian cancer compared with controls (11). Additionally, oral contraceptive use, which is inversely associated with ovarian cancer risk, suppresses testosterone production by 35% to 70% (1, 2).

Two highly polymorphic trinucleotide repeat polymorphisms in exon 1 of the AR gene have been studied in relation to cancer risk (12). The CAG trinucleotide repeat of AR encodes a polyglutamine tract (13), the length of which has been shown to be inversely associated with the ability of the AR-ligand complex to transactivate androgen-responsive genes. Molecular analyses have shown that the transactiva-

tional capacity of the AR decreases with increasing number of glutamines encoded by the CAG repeat tract (14). Indeed, shorter AR CAG repeat lengths are associated with a higher risk of prostate cancer (15). Racial differences in AR CAG repeat length have been noted, with African Americans having a lower mean CAG length as compared with Caucasians (16, 17). A second AR GGC trinucleotide repeat polymorphism codes for a polyglycine tract of variable length (18) but its functional significance has not been extensively examined. Data from one study suggest that whereas AR transactivation activity may not be affected by GGC repeats, translation of AR mRNA may be inversely related to GGC repeats with increased AR protein produced from alleles with shorter GGC repeats (19). This suggests that shorter GGC repeats may result in an increased capacity to respond to androgen exposure.

There are five published studies that have addressed the association between CAG repeat length and ovarian cancer. Two case-control studies reported an increased risk of ovarian cancer associated with increasing CAG repeat length among Caucasian women (20, 21). The data from both studies suggest that women who carry two alleles with ≥ 22 CAG repeats are more likely to develop ovarian cancer than those with two alleles with < 22 repeats. However, other published studies have not found evidence to support the association between longer CAG repeat length and ovarian cancer (12, 22, 23), although there may have been little power to detect an association due to the small number of ovarian cancer cases in two of the studies (12, 23). Kadouri et al. (12) also examined the relationship between GGC repeat length and 29 ovarian cancer cases and did not find evidence to support a relationship.

In view of the conflicting data about the relationship between AR repeat polymorphisms and ovarian cancer risk,

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Requests for reprints: Joellen M. Schildkraut, Duke University Medical Center, Box 2949, Durham, NC 27710. Phone: 919-681-4761; Fax: 919-681-4766. E-mail: schil001@mc.duke.edu

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we examined this relationship in a large population-based, case-control study of ovarian cancer in North Carolina. In contrast to prior studies, change point statistical analysis was used to determine the appropriate threshold for dichotomizing repeat lengths. In addition, this is the first study to examine the relationship between AR repeat length polymorphisms and ovarian cancer risk in a relatively large group of African American women. This is of interest because African Americans have shorter CAG repeat lengths relative to Caucasians, which might mediate an increased respond to androgen exposure (16).

Materials and Methods

Subjects. Study subjects were enrolled through the ongoing North Carolina Ovarian Cancer study, a population-based, case-control study of newly diagnosed epithelial ovarian cancer. Epithelial ovarian cancer cases were identified through the North Carolina Central Cancer Registry, a statewide population-based tumor registry, using rapid case ascertainment. Pathology reports for all ovarian cancer cases diagnosed in the study area were forwarded to the North Carolina Central Cancer Registry and then to the study office within 2 months of diagnosis. Eligibility criteria for ovarian cancer cases include diagnosis since January 1, 1999; age 20 to 74 years at diagnosis; no prior history of ovarian cancer; and residence in a 48-county area of North Carolina. For data included in the current analyses, the last diagnosis among Caucasians was November 2003. To maximize the number of African American subjects, the last date of diagnosis was extended to October 2005. All participants were English-speaking, mentally competent to complete an interview, and able to give informed consent. Physician permission was obtained before an eligible case was contacted. All cases underwent standardized pathologic and histologic review by the study pathologist to confirm diagnosis. Both invasive and borderline epithelial ovarian cancer cases were included. The response rate among eligible cases was 75%. Nonresponders were classified as patient refusal (7%), inability to locate the patient (9%), physician refusal (4%), death (4%), or debilitating illness (2%).

Population-based controls were identified from the same 48-county region as the cases and were frequency matched to the ovarian cancer cases on the basis of race (African American and Caucasian) and age (5-year age categories) using list-assisted random digit dialing. As required for the cases, controls had to be English-speaking, mentally competent to complete an interview, and able to give informed consent. Potential controls were screened for eligibility and were required to have at least one intact ovary and no prior diagnosis of ovarian cancer. Seventy-three percent of controls identified by random digit dialing, who passed the eligibility screening, agreed to be contacted and sent additional study information. Among those who sent additional study information, the response rate was 64%. Nonresponders were classified as refusal, 27%, and unable to contact, 9%. The study protocol was approved by the Duke University Medical Center Institutional Review Board and the Human Subjects committees at the North Carolina Central Cancer Registry and each of the hospitals where cases were identified.

Questionnaire Data. Trained nurse interviewers obtained written informed consent from study subjects at the time of the interview, which was usually conducted in the home of the study subject. A 90-min questionnaire was administered to obtain information on known and suspected ovarian cancer risk factors including family history of cancer in first-degree and second-degree relatives, menstrual characteristics, pregnancy and breast-feeding history, infertility, hormone use, and lifestyle characteristics such as smoking, alcohol consumption, physical activity, and occupational history. A life events

calendar, which marked significant life events including marriage and education, was used to improve recall of reproductive and contraceptive history. Additionally, anthropometric descriptors (height, weight, waist and hip circumference) were measured and a blood sample (30 mL) was collected.

Laboratory Analyses

DNA Extraction. Germ line DNA was extracted from peripheral blood lymphocytes using PureGene DNA isolation reagents according to manufacturer's instructions (Gentra Systems, Minneapolis, MN).

AR Trinucleotide Repeat Length Analysis. Thirty nanograms of genomic DNA were used as template for PCR amplification of the region containing the CAG and GGC trinucleotide repeats in 25- μ L reaction volumes. The CAG repeat was amplified using primers previously reported (12) with the exception that the forward primer was modified by the addition of a 5' fluorescent label (6-carboxyfluorescein; 6-FAM). The GGC repeat was amplified using two rounds of PCR with primers as described (24). For the GGC repeat analysis, the forward primer used in the second round of PCR was labeled with 6-FAM.

PCR for the CAG repeat was done using Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) with conditions as follows: 94°C for 3 min, then five rounds (four cycles each) of 94°C for 30 s, 64°C for 30 s for round 1, then decreased by 2°C each round down to 56°C, 72°C for 30 s, followed by 29 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, followed by a final 5-min extension at 72°C. For the GGC repeat, Pfu DNA polymerase (Stratagene, La Jolla, CA) was used with PCR conditions as follows: round 1, 98°C for 45 s, then 17 cycles of 98°C for 1 min and 70°C for 5 min, then a 10-min extension at 70°C. One microliter of the first-round PCR products was used as template for the second round of PCR under the same conditions except that 34 cycles of PCR were done.

The PCR products for both repeats were diluted 1:100 in nuclease-free water and these dilutions were run on an Applied Biosystems 3100 Automated Capillary Instrument followed by fragment analysis using GeneScan Analysis software (Applied Biosystems; Foster City, CA). To independently validate the fragment length call, a subset of samples were also analyzed by nucleotide sequencing after purification from high-resolution agarose gels of individual amplicons produced from each allele. Unlabeled forward primers were used for sequencing the amplicons using an ABI 3730 Prism capillary DNA sequencer for the CAG repeat ($n = 6$) or, for the GGC repeat ($n = 15$), the ThermoSequenase Radiolabeled Terminator Cycle Sequencing Kit (U.S. Biochemical Corp., Cleveland, OH). The GGC sequencing reactions were resolved on denaturing 5% polyacrylamide sequencing gels followed by exposure at -80°C to Kodak BioMax MR radiographic film with an intensifying screen. The length of the trinucleotide repeats by sequencing was found to be longer relative to the repeat length determined by fragment length call of the GeneScan software in all cases. Because the sequencing results provide direct visualization of the number of repeats present, we systematically adjusted the GeneScan fragment lengths by the addition of 12.0 nucleotides (4 repeats) for the CAG repeat analysis and 8.4 nucleotides (2.8 repeats) for the GGC repeat analysis.

Statistical Analysis. We did a two-stage analysis of the association between repeat length and ovarian cancer. In the first stage, we used Bayesian model selection and model averaging to determine the weight of evidence in the data for each possible cutoff point in repeat length and to estimate an average (over threshold values) measure of association (25). This approach allows us to determine if the association is significant marginal to the choice of threshold and obviates the

need for a multiple comparisons adjustment. In the second stage, we fit multivariate models of association fixing the repeat length threshold to its most probable value a posteriori. The purpose of these second stage analyses was to verify that the observed associations between repeat length and ovarian cancer were not confounded.

In stage 1, separate Bayesian change point models were fit to the self-reported African American and Caucasian short and long CAG repeat alleles, CAG_S and CAG_L, respectively, and short and long GGC repeat alleles, GGC_S and GGC_L, respectively. The designation of CAG_S and CAG_L, as well as of GGC_S and GGC_L, reflects the comparison of the repeat length of the two alleles within an individual. The change point model specifies that odds of disease is constant before and after a threshold value, but is different in the two regions. We used uniform prior probabilities over the possible discrete thresholds of the data and on whether or not there is a change point and used a $\beta(2, 2)$ prior over pre- and post-threshold probabilities of disease. The $\beta(2, 2)$ distribution has a mean of 0.5 and a SD of 0.22. This is equivalent to adding two cases and two controls in each of the pre- and post-threshold samples. Under this model, we calculated (a) the probability that there was a change point and, given that there was, (b) the probability that it occurred at each of the possible values. Calculation (a) was equivalent to a Bayesian hypothesis test of H_0 : the case control fraction does not depend on a thresholded short CAG repeat length versus H_a : that it does. We report the posterior probability for association of disease to CAG_S length, the posterior probabilities of the thresholds given that the threshold model is the true model, and the odds ratio (OR) for disease given a CAG_S repeat smaller than each probable threshold. In addition, we calculate estimates of ORs that account for uncertainty in the threshold value. This was accomplished by summarizing the marginal (over threshold) posterior distribution on the OR for the association between repeat length and ovarian cancer. The resulting OR is a threshold independent measure of association. Parallel analyses were calculated to determine the relationship between the CAG_L, GGC_S, and GGC_L repeat alleles in the AR gene and ovarian cancer risk.

The stage 1 change point models were fit under the assumption that there was no potential confounding by other covariates. In our stage 2 analysis, we checked this assumption by fitting multivariable unconditional logistic regression models controlling for potential confounders to determine whether confounding bias would explain any observed association between CAG and GGC repeat length and epithelial ovarian cancer. We examined the CAG_S and CAG_L repeat polymorphism variables using the cutoff points identified through the change point analysis as having the highest probabilities. Additionally, because of evidence from prior reports, we present the association between the number of CAG repeats ≥ 22 and ovarian cancer risk among Caucasian study participants. To control for confounding, we simultaneously adjusted for variables with known associations with disease status. These variables included age at diagnosis/interview, tubal ligation (yes or no), months of oral contraceptive use, body mass index (BMI; kg/m²) 1 year before diagnosis/interview, waist-to-hip ratio, family history of breast or ovarian cancer in first-degree relatives (yes or no), and total months pregnant. We report both age-adjusted ORs and 95% confidence intervals (95% CI) as well as ORs adjusted for additional potential confounders.

An interim analysis after the first 3 years of data collection revealed a statistically significant association with the CAG repeat polymorphism, but no evidence of an association with the GGC polymorphism. We therefore discontinued the analysis of the GGC repeats in this data set. Thus, our final sample size for the CAG polymorphism is approximately twice as large as that for GGC. For much of our sample, we had

additional genotype data on 99 unrelated single-nucleotide polymorphisms unlinked to disease status and selected from 22 chromosomes. Among the self-reported African American subjects, we had this genotype data on 77 of 99 cases and 88 of 141 controls. Among the self-reported Caucasian subjects, we had this genotype data on 473 of 495 cases and all of the controls. To address the possibility of population-stratification, the genotype data from these 99 single-nucleotide polymorphisms along with the location of each single-nucleotide polymorphism within its chromosome were input into the program Structure (version 2.0) to estimate the degree of racial admixture for each individual. Structure is a program that implements a model-based clustering method for inferring population structure (26). It allows the user to select the number of parent populations represented in the sample. We set this variable to 2 to allow for African and European ancestral populations. Structure estimated the admixture fractions for each individual. These admixture fractions were also used as an alternate to self-reported race status performing race-specific change point analyses in stage 1. Change point analyses were done using R.⁸ All other analyses were done using SAS 9.1 (SAS Institute, Inc., Cary, NC).

Results

The demographic features, epidemiologic risk factors, and pathologic characteristics of cases and controls are shown in Table 1, stratified by self-reported race. Tubal ligation was the only risk factor that had a statistically significant association in both races, in which an inverse relationship is observed. Waist-to-hip ratio was higher in cases than in controls both in Caucasians ($P < 0.001$) and among African Americans ($P = 0.059$). Fewer months of pregnancy and months of oral contraceptive use are observed among cases compared with controls for both races although these differences are statistically significant among Caucasians only. Tumor behavior was invasive in 76% and borderline in 24% for both African American and Caucasian cases. The distribution of histologic subtype was similar in both racial groups.

The CAG repeat length distributions in cases and controls by race are found in Table 2. No differences in the mean CAG repeat length in the AR gene were detected for either the short or long repeat alleles in Caucasian cases and controls. Among African Americans, the mean CAG_S and CAG_L repeat lengths were lower among cases compared with controls. The mean CAG_S length among cases was 16.8 (SD, 2.6) compared with 18.0 (SD, 2.7) among controls ($P = 0.001$), and the mean CAG_L length for cases was 20.7 (SD, 2.9) compared with 21.4 (SD, 2.5) for controls ($P = 0.044$). Both the mean CAG_S and CAG_L lengths were both significantly lower among African Americans compared with Caucasians ($P < 0.001$).

Parallel analyses to determine the association between GGC repeat and ovarian cancer risk were conducted in a subset of the population, which included 186 and 213 self-reported Caucasian ovarian cancer cases and controls, respectively, and 59 and 67 self-reported African American ovarian cancer cases and controls (see Table 2). There was no evidence of differences in the mean GGC_S or GGC_L allele length between cases and controls in either racial group.

Change point analysis was used to determine appropriate cutoff points for CAG repeat length for both the short and long CAG repeat alleles, stratified by self-reported race, either African American or Caucasian. Cutoff points were detected for both CAG_S and CAG_L repeat alleles among African Americans only. Tables 3 and 4 present model probabilities associated with the relationship between ovarian cancer and

⁸ <http://www.r-project.org>

Table 1. Demographics and pathologic characteristics of ovarian cancer cases and controls from the North Carolina Ovarian Cancer Study by self-reported race

	Caucasians			African Americans		
	Cases (N = 495), n (%)	Controls (N = 540), n (%)	P	Cases (N = 99), n (%)	Controls (N = 141), n (%)	P
Age (y)						
20-49	160 (32)	191 (35)	0.664	39 (39)	50 (35)	0.720
50-64	222 (45)	196 (36)		44 (44)	65 (46)	
65-75	113 (23)	153 (28)		16 (16)	26 (18)	
Menopause status						
Pre/peri	180 (36)	225 (42)	0.081	42 (42)	62 (44)	0.775
Post	315 (64)	315 (58)		57 (58)	78 (56)	
Months pregnant						
0	83 (17)	59 (11)	0.004	8 (8)	7 (5)	0.425
1-8	26 (5)	19 (4)		4 (4)	10 (7)	
9-18	182 (37)	206 (38)		36 (36)	48 (34)	
19-36	177 (36)	215 (40)		37 (37)	51 (36)	
>36	26 (5)	40 (7)		14 (14)	25 (18)	
Oral contraceptive use (mo)						
None	165 (33)	161 (30)	0.050	40 (40)	60 (43)	0.134
<12	42 (8)	44 (8)		14 (14)	5 (4)	
12-36	107 (22)	120 (22)		23 (23)	35 (25)	
37-60	45 (9)	46 (9)		3 (3)	6 (4)	
>60	122 (25)	164 (30)		15 (15)	31 (22)	
User of unknown duration	14 (3)	5 (1)		4 (4)	4 (3)	
History of breast/ovarian cancer in first-degree relative						
Yes	89 (18)	87 (16)	0.440	28 (28)	24 (17)	0.037
No	406 (82)	451 (84)		71 (72)	117 (83)	
Tubal ligation						
Yes	122 (25)	164 (30)	0.040	32 (32)	75 (53)	0.001
No	373 (75)	376 (70)		67 (68)	66 (47)	
Polycystic ovarian syndrome						
Yes	2 (0)	4 (1)	0.688	0 (0)	2 (1)	0.513
No	493 (100)	536 (99)		99 (100)	138 (99)	
BMI 1 y before diagnosis/interview						
Quartile 1: <22.42	103 (21)	131 (25)	0.167	NA	NA	0.368
Quartile 2: 22.42-25.739	133 (28)	133 (25)		NA	NA	
Quartile 3: 25.74-29.759	104 (22)	132 (25)		NA	NA	
Quartile 4: >29.76	143 (30)	133 (25)		NA	NA	
BMI 1 y before diagnosis/interview						
Quartile 1: <27.341	NA	NA		23 (24)	34 (25)	0.368
Quartile 2: 27.341-30.33	NA	NA		17 (18)	33 (24)	
Quartile 3: 30.34-36.4	NA	NA		26 (27)	35 (26)	
Quartile 4: >36.4	NA	NA		31 (32)	34 (25)	
Waist-to-hip ratio at interview						
Quartile 1: <0.739	82 (17)	133 (25)	<0.001	NA	NA	0.059
Quartile 2: 0.739-<0.7871	103 (21)	134 (25)		NA	NA	
Quartile 3: 0.7871-<0.8351	152 (31)	134 (25)		NA	NA	
Quartile 4: >0.8351	150 (31)	134 (25)		NA	NA	
Waist-to-hip ratio at interview						
Quartile 1: <0.772	NA	NA		16 (16)	35 (25)	0.059
Quartile 2: 0.772-<0.828	NA	NA		25 (26)	35 (25)	
Quartile 3: 0.829-<0.876	NA	NA		20 (21)	35 (25)	
Quartile 4: >0.876	NA	NA		36 (37)	34 (24)	
Infertility, doctor diagnosed in female						
Yes	62 (13)	53 (10)	0.166	8 (8)	10 (7)	0.775
No	433 (87)	487 (90)		91 (92)	131 (93)	
Tumor behavior						
Borderline	117 (24)			24 (24)		
Invasive	378	(76)		75 (76)		
Tumor histology						
Serous	300 (61)				61 (62)	
Endometrioid	63 (13)				12 (12)	
Mucinous	48 (10)				11 (11)	
Clear cell	37 (7)				2 (2)	
Other	46 (9)			13 (13)		

CAG_S repeat length allele and CAG_L repeat allele, respectively, conditional on thresholded CAG length among African Americans and Caucasians. The tables present estimates of posterior model probabilities for each cutoff point. OR estimates and 95% CIs for the association between CAG repeat length allele and ovarian cancer for each cutoff point are also shown. Among African Americans, the posterior probability of a change point association in the AR CAG_S repeat allele is

~72% and the most likely threshold is between 15 and 16 with a posterior probability of 31% given that the change point class of models is correct. The OR for the association between CAG repeat length at the threshold between 15 and 16 is 2.77 (95% CI, 1.31-5.26). The posterior probability of a change point association in the AR CAG_L repeat allele is ~73% and the most likely threshold is between 18 and 19 with a posterior probability of 21%. In contrast, the data for Caucasian ovarian

Table 2. Mean and median CAG and GGC trinucleotide repeat length polymorphism lengths in ovarian cancer cases and controls enrolled in the North Carolina Ovarian Cancer study by self-reported race

	Caucasians			African Americans		
	Cases	Controls	P	Cases	Controls	P
CAG repeats	(N = 484)	(N = 522)		(N = 99)	(N = 140)	
CAG_S						
Mean (SD)	19.4 (2.3)	19.3 (2.2)	0.685	16.8 (2.6)	18.0 (2.7)	0.001
Median (range)	19.0 (6-25)	19.0 (5-25)		17.0 (8-23)	17.0 (10-26)	
CAG_L						
Mean (SD)	22.6 (2.6)	22.4 (2.5)	0.146	20.7 (2.9)	21.4 (2.5)	0.044
Median (range)	23.0 (15-34)	22.0 (15-32)		21.0 (15-29)	22.0 (14-27)	
GGC repeats	(N = 186)	(N = 213)		(N = 59)	(N = 67)	
GGC_S						
Mean (SD)	16.6 (1.53)	16.4 (1.86)	0.377	15.4 (1.74)	15.2 (1.77)	0.593
Median (range)	17.0 (6-18)	17.0 (6-18)		16.0 (9-18)	16.0 (9-18)	
GGC_L						
Mean (SD)	17.2 (0.89)	17.2 (1.04)	0.800	16.6 (0.83)	16.6 (0.97)	0.925
Median (range)	17.0 (14-19)	17.0 (12-20)		17.0 (14-18)	17.0 (14-18)	

NOTE: P values are from Student's *t* test.

cancer cases and controls do not support a change point model; for the AR CAG_S repeat allele, the posterior probability of this class of change point models is 31% and no threshold has a posterior probability exceeding 14%. For the CAG_L allele, it is 36% and no threshold has a posterior probability exceeding 13%.

We repeated the change point analysis in a subset of cases and controls defined as African Americans based on having admixture fraction <10% as determined from the Structure analysis. A total of 13 cases and 21 controls of the self-reported African Americans who had an admixture fraction ≤90% were omitted. An additional 22 cases and 52 controls of the self-reported African Americans were omitted from this analysis due to missing admixture information. In this analysis, the relationship between CAG_S length and ovarian cancer became even stronger, suggesting that admixture does not explain the observed association with ovarian cancer (data not shown). In this analysis, the posterior of a change point association was 0.871 and, given the association, the probability of a change point between 15 and 16 was 0.675. For the CAG_L, there was no evidence of an admixture effect. Similarly, we repeated the change point analysis among Caucasians, omitting those with an admixture of <10%, and found that the posterior probability of a threshold-based effect

of the CAG_S allele on risk was 0.337, which was not different from the probability of 0.314 that estimated when using self-reported race to identify Caucasians.

Change point analysis did not detect an association between ovarian cancer and GGC repeat length for either the GGC_S or GGC_L repeats among those whose self-reported race was African American, those whose self-reported race was Caucasian, or those limited to having an African American admixture fraction of >90%. In fact, the probability for a change point did not exceed 41% for either the GGC_S or GGC_L allele in any of these groups (data not shown). Given a prior probability of 50% in favor of such an association, this is evidence against association.

Additional multivariable analyses to determine whether confounding could explain the association between CAG repeat length and ovarian cancer among African Americans are presented in Table 5. The age-adjusted OR for the association between the CAG_S repeat length allele <16 and ovarian cancer was 2.8 (95% CI, 1.4-5.9) in African Americans. A similar relationship was found between the CAG_L repeat length allele <19 and ovarian cancer (age-adjusted OR, 2.5; 95% CI, 1.3-4.8). Having both a CAG_S repeat <16 and a CAG_L repeat <19 was associated with a 5-fold increased risk of ovarian cancer (age-adjusted OR, 5.4; 95% CI, 1.6-17.9). Also

Table 3. Model probabilities conditional on thresholded CAG_S length and ORs for CAG_S repeat length less than versus greater than a threshold *t* by self-reported race

CAG_S length	Caucasians (484 cases and 522 controls)		African Americans (99 cases and 140 controls)	
	Posterior Pr($T = t/\text{change}$)	OR (95% CI)	Posterior Pr($T = t/\text{change}$)	OR(95% CI)
7.5	0.110	1.62 (0.19-6.30)	NA	NA
9.5	NA	NA	0.029	4.26 (0.34-19.54)
10.5	0.098	1.80 (0.35-5.80)	NA	NA
11.5	0.099	1.91 (0.48-5.44)	0.029	2.91 (0.60-9.26)
12.5	0.106	1.96 (0.59-5.11)	0.067	3.98 (0.91-12.45)
13.5	0.134	2.03 (0.74-4.64)	0.072	3.05 (1.01-7.52)
14.5	0.053	1.26 (0.55-2.48)	0.029	2.12 (0.83-4.54)
15.5	0.048	0.88 (0.44-1.57)	0.306	2.77 (1.31-5.26)
16.5	0.039	0.91 (0.51-1.48)	0.053	1.84 (1.04-3.00)
17.5	0.030	0.89 (0.63-1.21)	0.045	1.79 (1.02-2.92)
18.5	0.017	1.02 (0.77-1.31)	0.156	2.15 (1.18-3.65)
19.5	0.019	0.94 (0.73-1.20)	0.049	2.02 (1.02-3.70)
20.5	0.025	0.90 (0.67-1.19)	0.030	2.09 (0.88-4.35)
21.5	0.025	0.93 (0.66-1.27)	0.025	2.28 (0.75-5.80)
22.5	0.047	0.82 (0.52-1.23)	0.041	3.74 (0.77-13.13)
23.5	0.070	1.50 (0.63-3.09)	0.024	3.68 (0.39-16.43)
24.5	0.081	1.40 (0.33-4.10)	0.024	3.66 (0.40-16.35)
25.5	NA	NA	0.020	2.15 (0.17-10.03)
Overall	0.314		0.724	

Table 4. Model probabilities conditional on thresholded CAG_L length and ORs for CAG_L repeat length less than versus greater than a threshold *t* by self-reported race

CAG length	Caucasians (484 cases and 522 controls)		African Americans (99 cases and 140 controls)	
	Posterior Pr(<i>T</i> = <i>t</i> /change)	OR (95% CI)	Posterior Pr(<i>T</i> = <i>t</i> /change)	OR (95% CI)
14.5	NA	NA	0.021	1.41 (0.10-5.93)
15.5	0.078	1.62 (0.18-6.28)	0.020	2.14 (0.24-8.35)
16.5	0.067	1.44 (0.24-4.80)	0.015	1.73 (0.43-4.79)
17.5	0.082	0.67 (0.17-1.71)	0.022	1.85 (0.81-3.69)
18.5	0.067	0.69 (0.33-1.26)	0.205	2.44 (1.24-4.41)
19.5	0.025	0.87 (0.57-1.29)	0.204	2.24 (1.22-3.80)
20.5	0.017	0.91 (0.68-1.20)	0.150	2.05 (1.18-3.34)
21.5	0.032	0.84 (0.65-1.08)	0.180	2.08 (1.20-3.39)
22.5	0.036	0.83 (0.65-1.06)	0.008	1.32 (0.72-2.23)
23.5	0.048	0.81 (0.62-1.04)	0.007	1.18 (0.61-2.12)
24.5	0.015	1.06 (0.77-1.43)	0.010	1.39 (0.56-2.99)
25.5	0.021	0.92 (0.61-1.34)	0.011	1.02 (0.34-2.45)
26.5	0.024	0.99 (0.58-1.58)	0.034	0.53 (0.08-1.74)
27.5	0.060	0.72 (0.36-1.27)	0.070	0.35 (0.03-1.27)
28.5	0.065	0.71 (0.27-1.49)	NA	NA
29.5	0.053	1.30 (0.35-3.48)	NA	NA
30.5	0.082	0.93 (0.13-3.24)	NA	NA
32.5	0.127	0.62 (0.05-2.35)	NA	NA
33.5	0.102	0.93 (0.07-3.84)	NA	NA
Overall	0.362		0.734	

shown in Table 5, simultaneously controlling for age, months pregnant, months of oral contraceptive use, BMI, family history of ovarian or breast cancers in a first-degree relative, and tubal ligation did not substantially change the relationship between CAG repeat length and ovarian cancer detected in the age-adjusted analyses. Additional analyses limited to invasive ovarian cancers as well as histologic subtype (serous, endometrial, and clear cell only) did not reveal any substantial differences in the relationship with CAG repeat length and ovarian cancers (data not shown). Although we did not detect evidence for a threshold in CAG repeat length in Caucasians, we calculated the age-adjusted OR for a CAG_S repeat length <16 of 0.8 (95% CI, 0.4-1.5) and for the CAG_L repeat length <19 of 0.6 (95% CI, 0.3-1.2).

We also conducted unconditional logistic regression analyses in Caucasian subjects using a cutoff point of ≥ 22 CAG repeats to compare our data to those of previously published reports (20, 21, 27). The age-adjusted ORs for the association between those who carry either one or two alleles with ≥ 22 CAG repeats versus those with two alleles with <22 repeats were 1.2 (95% CI, 0.9-1.6) and 1.2 (95% CI 0.8-1.7), respectively

Table 5. Relationship between AR CAG repeat polymorphisms and ovarian cancer among African American women enrolled in the North Carolina Ovarian Cancer Study

	Cases, <i>n</i> (%)	Controls, <i>n</i> (%)	OR* (95% CI)	OR [†] (95% CI)
CAG_S repeat <16				
No	76 (77)	126 (89)	1.0 (reference)	1.0 (reference)
Yes	23 (23)	15 (11)	2.8 (1.4-5.9)	2.5 (1.1-5.5)
CAG_L repeat <19				
No	72 (73)	121 (86)	1.0 (reference)	1.0 (reference)
Yes	27 (27)	20 (14)	2.5 (1.3-4.8)	2.7 (1.3-5.8)
No. with CAG_S <16 or CAG_L <19				
None	60 (61)	111 (79)	1.0 (reference)	1.0 (reference)
1 allele	28 (28)	25 (18)	2.2 (1.1-4.1)	2.1 (1.1-4.3)
2 alleles	11 (11)	5 (4)	5.4 (1.6-17.9)	4.8 (1.4-17.0)

*Age adjusted.

[†]Adjusted for age, months pregnant, months of oral contraceptive use, BMI, tubal ligation, family history of breast or ovarian cancer in a first-degree relative, waist-to-hip ratio.

(see Table 6). Restricting this analysis to Caucasian women of <10% admixture, an age-adjusted OR of 1.3 (95% CI, 0.9-2.0) was calculated for the association with two alleles with ≥ 22 repeats.

Discussion

The mean and median AR CAG lengths for both the CAG_S and CAG_L alleles in Caucasian subjects in the North Carolina Ovarian Cancer study population are similar to lengths reported in previous studies (20, 21, 27). No relationship was found between CAG repeat length and ovarian cancer among the Caucasians in this study. To our knowledge, this is the first study to evaluate the association between CAG repeat length in AR and ovarian cancer risk in African American women. We found an increase in ovarian cancer risk associated with both CAG_S and CAG_L repeat length alleles in African Americans. These differences were evidenced by both the shorter mean repeat length of the CAG_S and CAG_L alleles as well as the higher prevalence of the CAG_S repeat length <16 and the CAG_L repeat length <19 among African American cases compared with controls.

The association between AR CAG repeat length and ovarian cancer risk in African Americans is further supported by an analysis of these data that omitted self-reported African American subjects with evidence of significant admixture of

Table 6. AR CAG repeat polymorphisms ≥ 22 versus ≤ 22 repeats among Caucasian cases and controls in the North Carolina Ovarian Cancer study

	Cases, <i>n</i> (%)	Controls, <i>n</i> (%)	OR* (95% CI)	OR [†] (95% CI)
CAG repeat ≥ 22				
0 allele	163 (34)	198 (38)	1.0 (reference)	1.0 (reference)
1 allele	237 (49)	240 (46)	1.2 (0.9-1.6)	1.2 (0.9-1.6)
2 alleles	84 (17)	84 (16)	1.2 (0.8-1.7)	1.2 (0.8-1.8)
Either 1 or 2 alleles	321 (66)	324 (62)	1.2 (0.9-1.5)	1.2 (0.9-1.5)

*Age adjusted.

[†]Adjusted for age, months pregnant, months of oral contraceptive use duration, BMI, tubal ligation, family history of breast or ovarian cancer in a first-degree relative, waist-to-hip ratio.

>10% and found a similar association. Therefore, this result is not likely to be explained by confounding due to population stratification. Additionally, our results remained significant when simultaneously controlling for other potential confounders. Our results suggest that having one short CAG repeat length in African Americans, which is associated with higher levels of androgenic activity, more than doubles the risk of ovarian cancer. Additionally, those with both short CAG_S and short CAG_L repeats may have a 5-fold increased risk of ovarian cancer. This is also the largest study to evaluate the relationship between the AR GGC repeat length polymorphism and ovarian cancer. Similar to the finding of a small case-control study by Kadouri et al. (12), we did not detect a relationship in either racial group.

The observed association between CAG repeat length and ovarian cancer is biologically plausible in view of the inverse relationship between CAG length and transactivation activity on the receipt and binding affinity of androgens (14). Short CAG repeat alleles may facilitate greater chronic androgen stimulation leading to increased proliferative activity. Shorter CAG repeat alleles have also been associated with other hyperandrogenic clinical conditions including risk of baldness and having prostatic hyperplasia in men and hirsutism (28), anovulation (29), and acne in women (30). Additionally, data from a nested case-control study by Helzlsouer et al. (11) found that increased serum androgen levels were associated with an increased risk of ovarian cancer.

Change point analysis of our data does not support a choice of a threshold. However, two recent studies by Terry et al. (20) and Santarosa et al. (21) support an association between having two alleles with ≥ 22 CAG repeats and ovarian cancer risk in Caucasian subjects. The relationship in the study by Santarosa et al. was stronger, with an OR of 3.45 (95% CI, 1.42-8.34) compared with 1.31 (95% CI, 1.01-1.59) in the study by Terry et al. (20). For comparative purposes, we did analyses among Caucasians using the cutoff point reported in these studies and found an OR of 1.2 (95% CI, 0.9-1.6) for self-reported Caucasian and 1.3 (95% CI, 0.9-2.0) among those with <10% admixture. These ORs, although weak, are similar in magnitude and precision as that reported by Terry et al. (20). As suggested by Terry et al., a possible explanation for the discrepancies between previously published reports that may also explain the findings in the current study includes differences in the prevalence in the carriage of subjects having two AR CAG repeats ≥ 22 . It is known that allele frequency varies according to ethnicity (16, 31). In our study, we found that the prevalence of two CAG repeats ≥ 22 differed markedly between Caucasian and African American controls, 16% and 9%, respectively. The prevalence of two AR CAG repeats >22 in our Caucasian subjects also differs from the prevalences among Caucasians in the studies by Terry (prevalence, 24%) and Spurdle et al. (prevalence, 26%), a positive and a negative study, respectively, but is more similar to that of Santarosa et al. (prevalence, 18%), which was a positive study. Due to the known ethnic variation in CAG repeat length, it is possible that the results of the association with AR CAG length could be due to chance. However, our analyses of admixture among the Caucasian subjects did not support that population stratification was a major concern; only 8% of cases and 11% of controls had evidence of significant (>10%) admixture, and when we reanalyzed our data, omitting subjects with >10% admixture, our findings did not change.

Strengths of this study include the fact that this is a large population-based study of both Caucasian and African American women. Our approach using the change point analysis provided a more objective and thorough evaluation of a cutoff point in the association between AR CAG repeat length and ovarian cancer risk, avoiding multiple comparisons at different thresholds. The nature of the change point analyses sidesteps the issue of multiple comparisons. In this approach,

we simultaneously calculate (a) the posterior probability of a change point association versus the alternative of no association and (b) the posterior probability of each of a discrete set of thresholds being the location of the change point given that there is one. This decouples the question of whether or not there is a change point association from the question of the appropriate threshold. In addition, we estimated the association between AR CAG repeat length while simultaneously controlling for other potential confounders, thus providing evidence that confounding bias is unlikely to account for the association. We were able to determine that it was unlikely that population stratification among African Americans biased our results. It is also unlikely that selection bias related to genotype would have occurred and influenced our results. Limitations of our study include a somewhat small sample of African American subjects. We attempted to find an independent data set that could be used for a validation of the association between AR CAG repeat length and ovarian cancer in African Americans, but we were unsuccessful. Finally, we were not able to conclusively determine why the findings among African American women and Caucasian women differed. The failure to observe the relationship in Caucasians may be due to the rarity of the short CAG alleles in this population or could reflect racial differences in disease etiology. For example, compared with Caucasian women, differences in the prevalence of other genetic variants and other characteristics, such as BMI and waist-to-hip ratio, among African American women may play a role. Further exploration of these factors may help increase the understanding of ovarian cancer etiology.

Similar to studies in ovarian cancer, analyses of the relationship between the short AR CAG repeat length polymorphism and prostate cancer risk also have yielded conflicting results. Likewise, differences in the association with prostate have been noted between racial groups (17, 32-35). Pettaway (32) has suggested that racial differences in genetic variation in several genes in the androgen/AR pathway may be related to clinically observed differences in the biology of prostate cancer among racial groups (32). For example, in addition to CAG repeat length in AR, genetic variants in the 5 α -reductase type 2 also differ between African Americans and Caucasians. However, it has also been suggested that racial differences and inconsistent findings in studies of prostate cancer may be due to linkage disequilibrium between AR CAG repeat length polymorphisms and another susceptibility locus on the X chromosome (36). These possible explanations are also relevant to studies of ovarian cancer.

In summary, our finding of an association between short AR CAG repeat lengths and ovarian cancer among African Americans warrants replication in a larger data set, and further study is needed to more fully understand the complexities of this relationship. We did not detect a relationship between CAG repeat length and ovarian cancer among Caucasian women and were not able to confirm previous reports for such an association. Additionally, we were unable to detect a relationship between the GGC repeat polymorphism and ovarian cancer in either African American or Caucasian women. However, we believe that further study of the positive finding in African American women may provide insight into the etiology of ovarian cancer.

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